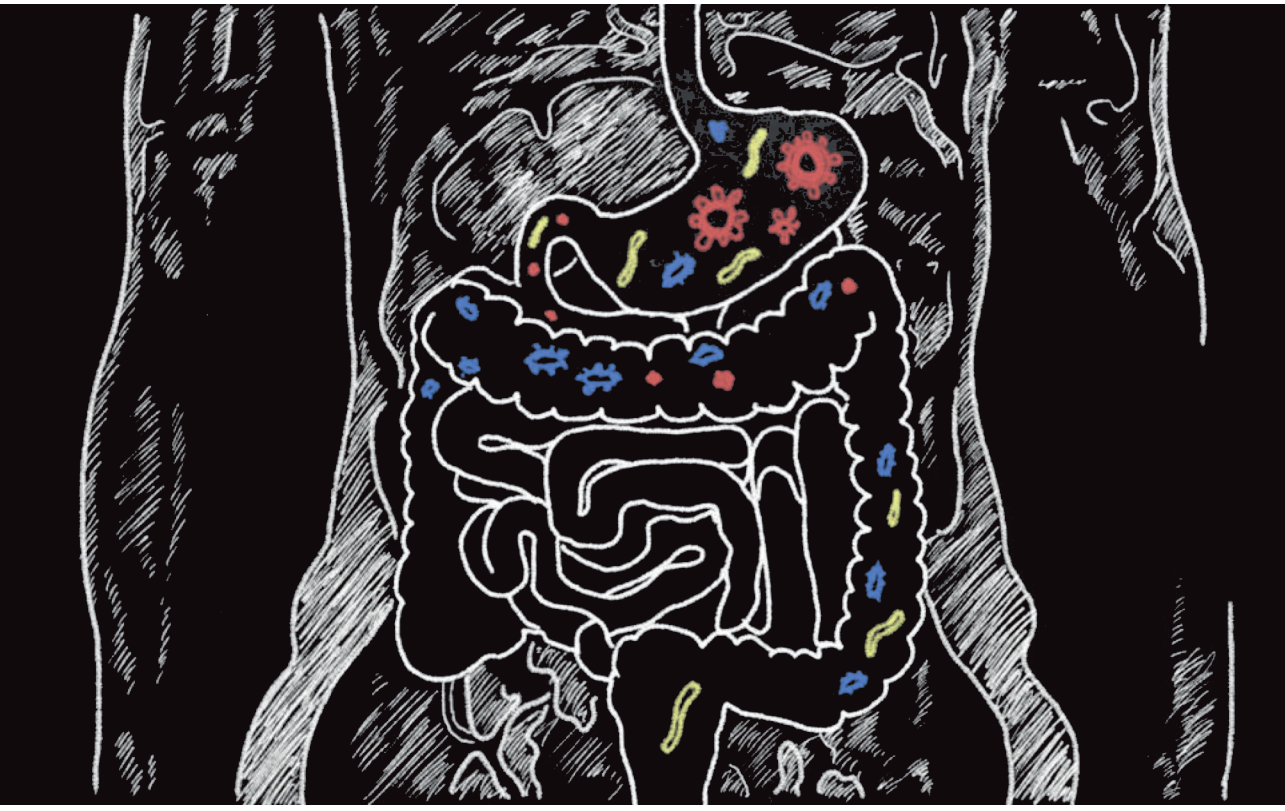
HELICOBACTER PYLORI AND GASTRIC CANCER:

From Tumor microenvironment to Immunotherapy



Adamu Ishaku Akyala

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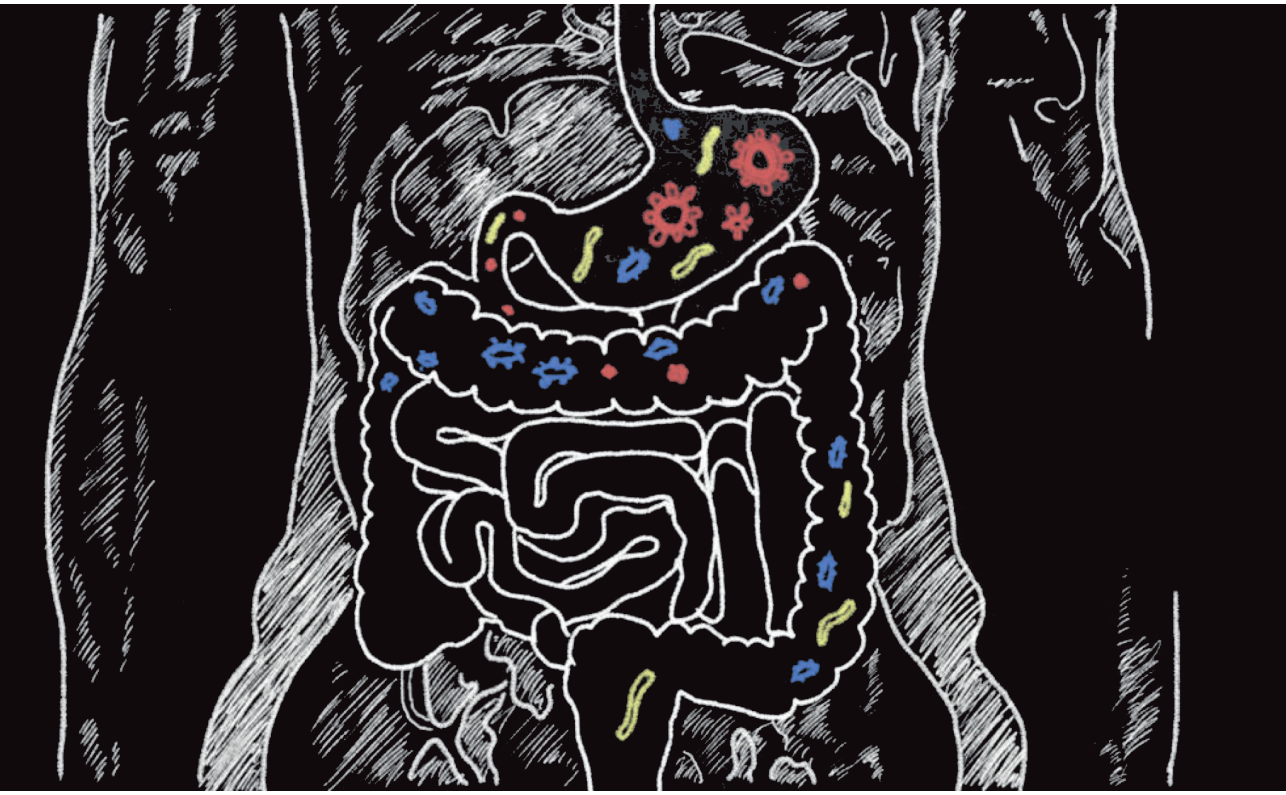

Chapter 1

General Introduction, aims and outline

Cancer

In 400 BC, Hippocrates, the Greek physician generally regarded as the “father of Medicine” was perhaps the first to recognize cancer as a separate disease entity which he denominated as “carcinos”. In Greek, the word refers to a crab. This invertebrate was probably associated with the disease because the finger-like spreading projections emanating from a growing cancer provoke associations with the morphological aspect of a crab. Referring to this concept of ‘carcinos,' as a disease entity in 28-50 BC, a Roman physician by the name of Celsus was probably the first to translate the Greek crab term to the Latin “Cancer” and this has been the name of this group of diseases ever since. Despite millennia of effort, however, the clinical problems associated with preventing and curing cancer have not been solved.

In our time, a group of over a thousand diseases share the generic name cancer. We recognize different types of cancer but all arise from unusual properties acquired by the cells involved. Once established, cancers often affect a different part of the body as from whence they originate and involve the loss of the intrinsic mechanisms that inhibit cell proliferation. Multiplication of cells is an intricate process requiring elaborate biochemical coordination. In physiology compartment size is well-controlled and limited by various forms of cell death, control from which cancer cells escape. In addition, tumor cells escape from the immune system surveillance, and thus instead of being killed, they proliferate rapidly at a geometric rate forming a mass of cells; a tumor. Also, cancer cells survive and multiply outside their normal tissue boundaries, invading neighboring tissues. Furthermore, cancer cells are highly mobile within the body and can affect different parts of the body. Following seeding they can initiate growth of a new cancer that can affect healthy tissue. Upon entrance of cancer cells into the lymph and bloodstream, migration to a different part of the body is even further enhanced. The process of cancer spreading, i.e., metastasis, is the primary cause of mortality due to cancer. In this thesis I want to contribute to the battle against this disease.

The urgency of such efforts is illustrated by that oncological disease accounts for annually 8.2 million cancer-associated deaths (2012 numbers) and 14 million newly diagnosed patients. Victories against other types of mortality now also make cancer an emerging global public health problem(1), a development also occurring in Nigeria, my home country. Most prevalent cancer types include the gastrointestinal,

prostate, liver, lung, breast, cervix, colorectal manifestations (2). Cancer can arise from any form of body tissue and occurs when a typical cell acquires six core biological hallmarks which turn it into a tumor cell. Hanahan et al.(3, 4), describe these six core hallmarks of cancer to include the ability of tumor cells to: 1) sustain continuous proliferation through constitutive activation of proliferative signaling pathways or disruption of negative feedback mechanism on cell proliferation, 2) to evade growth suppression through the inactivation of tumor suppressor genes, which in normal physiology generally turn of an increased signaling and control the cell cycle progression, 3) to resist cell death by the loss of pro-apoptotic factors and the upregulation of anti-apoptotic factors, 4) to enable replicative immortality through the extension of the telomeric DNA at the ends of the chromosome by telomerase enzymatic activity, 5) to stimulate angiogenesis to provide access of the tumor to nutrients and oxygen and for removal of metabolic wastes, 6) to undergo invasion and metastasis whereby tumor cells alter their shape and lose their adherence to other extracellular matrix and cells (Fig. 1A)[(3)].

These six 'core' hallmarks are somewhat artificial as it is now becoming evident that tumor cells can also acquire other typical hallmarks of which reprogramming of their energy metabolism, using glycolysis to produce energy even under aerobic conditions, is maybe the most evident. The latter are sometimes denominated as "emerging hallmarks."

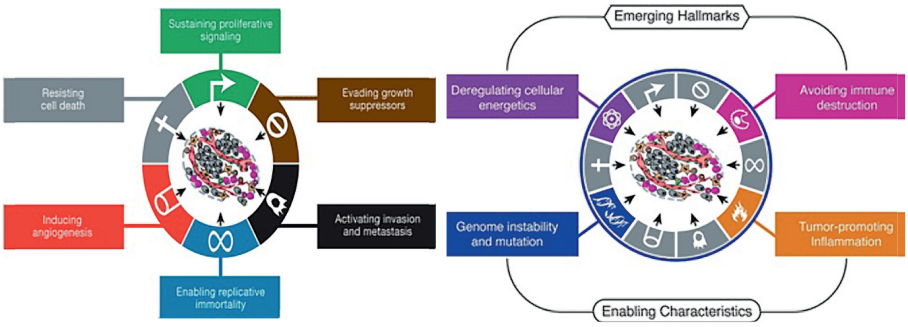

Figure 1. The cancer hallmarks (A) Schematic depiction of the six 'core' properties typically acquired by a tumor cell during its multistep development. These six properties are sustaining proliferation, evading growth suppression, resisting cell death, enabling replicative immortality, inducing angiogenesis, and activating invasion and metastasis. (B) Furthermore, tumor cells acquire two emerging hallmarks, reprogramming energy metabolism and avoiding the immune system, and two enabling hallmarks, genome instability, and tumor-promoting inflammation. (Adapted from reference)(3)

In this context, it is also important to mention the potential to escape immune system surveillance. Furthermore, the accumulation of mutation and genome instability results in an ever-expanding expansion of subclones of the tumor cells, which will be subject to selection (also in response to therapy). As mentioned many tumors promote inflammation, causing normal immune cells to infiltrate and also attack the tumor cells. But this beneficial effect is often counterbalanced by the resulting inflammation-dependent promotion of tumor growth, activation of pro-survival pathways and release of proangiogenic factors (Fig. 1B)[3]. A block of differentiation can be distinguished as another hallmark of cancer, and this is caused by the ectopic expression or loss of key transcription factors. In this thesis, I try to take all of these factors into account in an effort to explain the cancer process.

In essence, genetic and epigenetic alterations are the underlying causes for these cancer hallmarks to appear. These epigenetic changes prominently include DNA methylation and covalent histone modifications, whereas the genetic changes involve processes such as deletion, amplification, insertion, translocation or point mutations, in addition to changes in the expression levels of the affected genes or changes in enzymatic activity. In conjunction, these changes provoke the appearance of the above-described cancer hallmarks. The genetic alterations can be inherited or acquired due to exposure to environmental factors, like tobacco smoking, or can be associated with specific dietary habits (high-fat), contact with toxic chemicals or viral and/or bacterial infection. If these genetic changes provide the cells with a selective advantage, this results in their outgrowth within the local tissue. Further accumulation of genetic changes because of genome instability, can then promote tumor cells to acquire increased invasive and metastatic potential[2]. It is, however, fair to say that in many cases we only poorly understand how all these effects conspire to provoke disease.

Understanding cancer thus represents one of the significant challenges for the scientific community in the present century. Some types of cancer have a more substantial impact on public health as they present with higher incidence and mortality, among them gastrointestinal cancer. In the general introduction of this thesis, I present an overview of the current knowledge on gastric cancer, addressing epidemiological, clinicopathological and biological aspects.

Gastric cancer

Gastric cancer remains a significant public health problem globally, and it is among the leading causes of cancer-associated mortality world-wide(5, 6). Although only 25–30% of gastric cancer patients will survive the five years following diagnosis, substantial heterogeneity of survival rates is reported in clinical trials, and this may relate to variations in the biological and genomic make-up, especially when Western and Asian populations are compared(7). Gastric cancer incidence dramatically varies across different geographical areas, being the highest in Japan, China, Far Eastern countries, Russia, Middle Eastern area, and in the Pacific coast of the South American continent and the being the lowest in Central Africa. Europe and North American regions share an intermediate-to-high incidence(8). Stomach and breast cancers have the highest incidence rates of all other cancers and are for instance among the most common causes of death in Iran(8). Conversely; there are low prevalence and incidence rates in sub-Sahara Africa(9-13). Gastric cancer patient's prognosis is a function of disease stage progression, in which less than 20% progress to advanced stages in patients surviving five years following diagnosis. It appears important to individualize treatment and screening strategies for the high-risk groups. Several risk factors are responsible for the progression to advanced stages of gastric cancer(14-17). Gastric cancer risk is associated with *Helicobacter pylori* infection which accounts for 70% cancer incidence in the stomach, and accordingly, the eradication of this bacteria provides primary prevention for cancer development(2, 18). Generally, one can say that family history, intestinal metaplasia, salt intake, smoking, alcohol and *H pylori* infection are the most significant risk factors for developing the premalignant conditions that ultimately give rise to gastric cancer development(19-22). Japan and South Korea adopted a strategy of early detection among other approaches, which has resulted in a favorable decrease in incidence and prevalence rate. The median survival rate of full-blown gastric cancer is only 9 to 10 months. There is hope that profiling of immune and molecular details of the disease, as well as the introduction of immune checkpoints inhibitors, may increase the overall survival rate and the present thesis also aims to contribute in this respect.

Descriptive epidemiology of gastric cancer

Global estimate: incidence and mortality

The Global Burden of Cancer Study (GLOBOCAN 2008)(18) provides the most recent figures available for the worldwide cancer burden with 988,000 new gastric cancer cases accounting for 7.8% of the total global cancer burden. This makes gastric cancer the fourth most prevalent global cancer after lung cancer (1.68 million cases; 12.7% of all cancers), breast cancer (1.31; 10.9%), and colorectal cancer (1.24; 9.8%). More than 73% (728,000 cases) of gastric cancer cases occur in parts of Asia, and almost half the world's total (47%) of gastric cancer occurs in China (Fig. 2). Europe contributes nearly 15% of to the global burden (146,000 cases) of gastric cancer, whereas Central and South America add a further 7% (65,000 cases) (see Fig. 2).

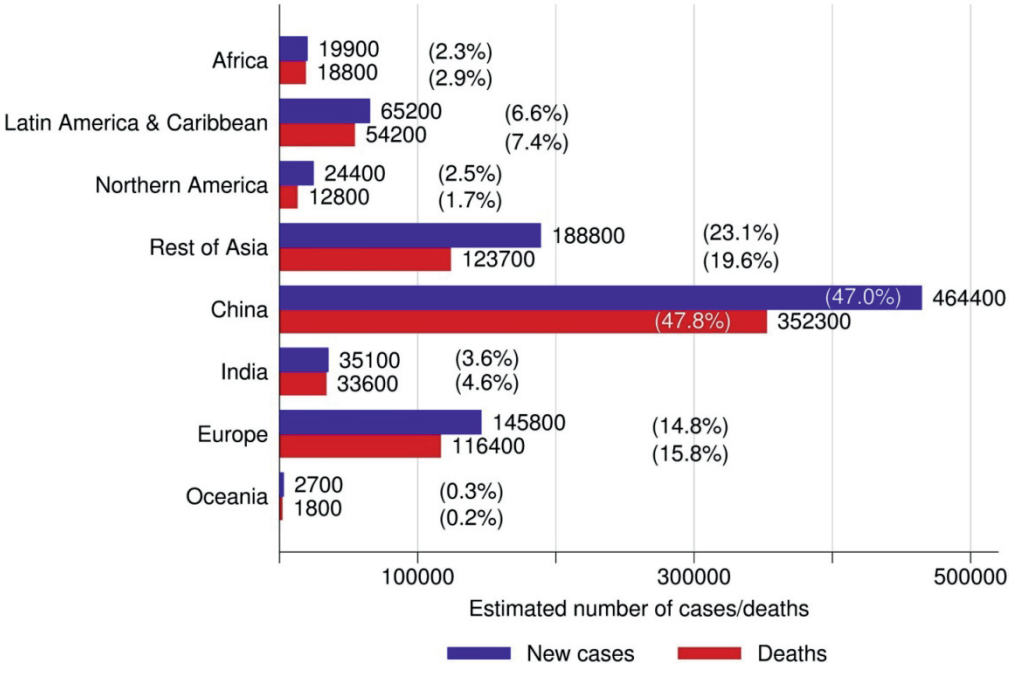

Fig. 2. Mortality and new cases estimated number of global fraction proportion of gastric cancer. Available at: http://globocan.iarc.fr. Accessed January 7, 2013.)

Prognosis following a gastric cancer diagnosis is usually poor. This is evident from the pattern of mortality which shows great similarity that of the incidence of gastric cancer,, and the proportional breakdown of the key two indicators by continent does not seem substantially different (see Fig. 2). The above estimates represent the figures for cases of the histological type of cancer known as gastric adenocarcinoma, but other types of gastric cancer exist as well. Several studies recently estimated a worldwide total of lymphomas of gastric origin in 2008 to be 18,000 (i.e., less than 2% of the number of adenocarcinomas)(23). Other gastric malignant histologies are even less frequent than this. Hence in this thesis, I focus on the adenocarcinoma of the stomach.

A recent study by Hu *cum suis* indicated a relationship between metabolic syndrome and increased risk for gastric adenocarcinoma(24). The main finding of Hu and colleagues is an important issue, not only for clinicians who are faced by gastric cancer patients but also for health policy makers in Asian countries, who are challenged with the burden of gastric cancers in their populations. We know that the problem of gastrointestinal cancer is growing due to aging populations, smoking, obesity, and changing lifestyle in Asia(25, 26), which are all associated with metabolic syndrome. Thus controlling metabolic syndrome will remain essential for managing the cancer burden in the Asian population. As I am skeptical that important improvements in this respect will be made anytime soon, I feel that the need to develop more insight into gastric cancer pathogenesis and to develop new modes of treatments will remain necessary and this what I intend to aid through this thesis. I shall focus on *H. pylori* as a risk factor, the role of endoscopy in diagnosis, the potential of targeting Hedgehog signaling and the potential of checkpoint inhibitors in gastric cancers. The reason for these choices I shall try to explain below.

Risk factors for gastric cancer

Helicobacter pylori

It is assumed that *H. pylori*-infected individuals were once ubiquitous in the human population, but in many populations, the prevalence is successively declining as determined in birth cohorts and it is rare among children in Japan, Western Europe, North America, and Oceania(27). Risk factors associated with *H. pylori* infection, and thus gastric cancer, include overcrowding, poor sanitation and low

socioeconomic status(28, 29). The relationship *H. pylori* infection and gastric cancer remained controversial for some time until it was effectively ended in 1994, when an expert (IARC) working group convened and classified *H. pylori* infection as a carcinogen to humans, based on its association with gastric cancer and mucosa-associated lymphoid tissue lymphoma (30). Although not really subject to debate anymore, this hypothesis was confirmed in 2009 by yet another second IARC working group(31), with the added precision that *H. pylori* causes noncardia gastric carcinoma thus implying that infection with *H. pylori* is not a risk factor for gastric cancer in its totality but restricted to the distal part of the stomach. A study recently estimated that 75% of noncardia gastric are associated with *H. pylori* infections(31). However, it now seems that the relationship between *H pylori* and gastric cancer may even have been underestimated, because of inaccurate assessment of *H. pylori infection* status. Indeed, it has been hypothesized the necessary causal factor for gastric cancer is *H. pylori* infection(32). Almost all of the epidemiologic evidence on the relationship between gastric cancer and *H. pylori* comes from a serologic assessment of *H. pylori* IgG. It is now widely accepted that retrospective serologic evaluation of gastric cancer with *H. pylori* infection cases has poor sensitivity so that case-control studies systematically underestimate the strength of the association. Atrophic gastritis causes this problem, a precancerous lesion, which leads to a reduction in infection burden to *H. pylori* and a subsequent decrease in IgG antibody titers to *H. pylori*, which may become serologically undetectable. For this reason, most of the evidence for the carcinogenicity of *H. pylori* is from prospective studies. The most comprehensive comparative risk estimates for *H. pylori* association with gastric cancer come from 12 prospective studies pooled analysis, which included cases of noncardia gastric cancer (762) and controls (2250). The combined (OR) of *H. pylori* infection was 2.97(2.34–3.77)(33). The same author included 274 cardia gastric cancer cases with the same control (827,) with (OR) 0.99 (0.40–1.77) for *H. pylori* infection. When restricted cases in a pooled analysis of over ten years blood are drawn *H. pylori* diagnosis, an increased Odd ratio of 5.93 (3.41–10.3) for non-gastric cancer of noncardia origin but reduced odds ratio of 0.46(0.23–0.90) for cardia cancer. This subgroup analysis underscores both the difference between cardia and noncardia cancer and the need to account for the effect of gastric carcinogenesis on *H. pylori* measurement, even in prospective studies(31). It is important to note, however, that although *H. pylori* infection may explain most if not all gastric cancers, most people infected with the bacterium do not develop such cancer. Hence understanding what makes an *H. pylori*-dependent infection potentially hazardous is of utmost importance

The CagA pathogenicity island

The diversity of the *H. pylori* genome is responsible for the differences in clinical prognosis. *H.pylori* can differ in quite a number of genetic factors expressed upon stomach tissue colonization; these factors include the virulence factors VacA and CagA and BabA, SabA, alphAB and HopZ which have all been reported to be associated with progression towards gastric cancer. The *H. pylori* virulence factor CagA (cytotoxin-associated gene A) is a 120–145 kDa protein encoded on a 40 kb cag pathogenicity island (denominated as PAI) and considered as one of the most relevant pathogenicity factors in relation to progression to gastric cancer. *H. pylori* strains are thus subdivided into CagA positive or negative strains. Approximately half of the *H. pylori* strains isolated in Western countries contain cag/PAI, whereas almost all of the East Asian isolates are cag/PAI-positive(31). A case-control study involving 778 gastric cancers from the non-cardia origin and 1409 matched controls revealed excess *H. pylori* CagA positive strains in the gastric cancer group resulting in an odds ratio (OR) of 2.01 CI (1.21–3.32) progression towards gastric cancer(34). Mechanistically the cag pathogenicity island is associated with the establishment of precancerous gastric lesions, implying a role for the bacterium in the early phases of progression towards full-blown disease. Plummer and colleagues(35), analyzed the results of a cross-sectional endoscopic survey of 2145 individuals from Venezuela, in which both DNA and CagA gene of *H. pylori* gene were determined by polymerase chain reaction on gastric biopsies. Infection with *H. pylori* cagA-positive strains but not negative cagA-strains appeared associated with severity of the precancerous lesions in this study. Using individuals with normal gastric mucosa or superficial gastritis as controls, the OR for dysplasia was 15.5(6.4–37.2) for *H. pylori* cagA-positive strains compared to 0.90(0.37–2.17) for cagA-negative *H. pylori*. Gonzalez and colleagues (35), analyzed the results of a follow-up study of 312 individuals from Spain with 12.8 years as an average follow-up time between two endoscopies, also involving the results of polymer chain reaction detection of cagA and genotyping of the *H. pylori* results. The relative risk for progression of precancerous lesions was 2.28 (1.13–4.58) for cagA-positive strains compared to cagA-negative strains. It is clear. However, that cagA alone does not explain the presentation of gastric cancer in the population in relation to *H. pylori* and this thesis I endeavor to identify other markers differing between *H. pylori* strains that may explain alternative gastric cancer risk.

Other Risk Factors

Socioeconomic status (SES) and surrogate factors

Developing countries share a higher burden of gastric cancer than in the developed world. This appears due to differences in socio-economic status. In developed countries, like The Netherlands, both incidence and mortality are currently decreasing, due in part to the slow disappearance of *H. pylori* that accompanied the uninterrupted access to better living conditions in people born after World War II. Indeed, within any country or population, noncardia gastric cancer is most often seen in lower socioeconomic groups and has been associated with many risk factors that act as a surrogate for lower SES, mainly low income, lower education, number of siblings, crowding, and lower occupational activity(36, 37). Hence, higher SES is inversely associated with gastric cancer of noncardia origin, whereas cardia gastric cancer is strongly associated with esophageal adenocarcinoma and both are associated with a higher SES. Many other factors involved in gastric cancer epidemiology are also associated with SES and probably confound some of the observed associations, although adjusting for *H. pylori* infection in a large European multicenter study, makes the effect of SES in non-cardia gastric cancer entirely disappear(38). Thus, although other factors such as fruit and vegetable consumption, cigarette smoking, and physical activity, may also confound any observed association with SES, it is clear that *H. pylori* is a major driving force in the development of gastric cancer, justifying my efforts of linking specific substrains of this bacterium to clinical outcome of disease.

Tobacco and alcohol

In relation to the former, smoking is a recognized cause of gastric cancer but seems to act as a moderate risk factor, compared to other associated risk factors of gastric adenocarcinoma. A meta-analysis and systematic review, (including cohorts, case-cohorts, and nested case-control studies and other prospective studies) produced a risk estimate for gastric cancer of 1.62 (1.50–1.75) in male smokers and an assessment of relative risk of 1.20(1.01–1.43) in female smokers as compared to self-reported never smokers(39). Meta-analyses studies have furthermore revealed that the risk for stomach cancer increases with increasing cigarette consumption (as expressed either as average number per day, number of pack-years, or as a function of a longer period of smoking)(40). Nevertheless, as compared to for instance lung

cancer, the effects of smoking are small with respect to development of cancer in the stomach and thus I decided not to investigate this aspect in the studies described in this thesis, but focus on other factors in their relation to gastric cancer.

Food and nutrition

In 2007 an expert panel assembled by the American Institute for Cancer Research scored the risk for cancer development in relation to diet and nutrition on a five-tier scale and concluded that an association between diet and gastric cancer exists(41). According to this panel, protective effects of diet on the development of gastric cancer emanating from various studies could be scored mostly as level 1 (convincing) evidence, but a few studies were scored as level 2 (probably) evidence. Probable protective factors are abstaining from carbohydrate intake and ample consumption of vegetables and fruits. Likely risk factors are high salt intake and consistently salt-preserved foods. For other dietary factors, scores were level 3 (limited evidence). Again, I decided to ignore this aspect of gastric cancer pathogenesis in my studies, as I found that other aspects were more deserving of study.

Epstein-Barr Virus

H. pylori is not the only infectious agent associated with malignant transformation in the stomach. Epstein-Barr virus (EBV) is present in 10% of gastric cancers. There is strong mechanistic evidence that EBV can provoke gastric cancer. EBV DNA appears incorporated in cancer cells as detected by the presence of antigen in gastric cancer(42). However, the epidemiological evidence that shows an association between gastric cancer and EBV is weak, mainly because of the difficulty of controlling for the confounding infection with *H. pylori*. For this reason, the same working group which concluded that *H. pylori* causes noncardia gastric cancer did not conclude that EBV causes gastric cancer(42) and as a consequence of this analysis I did not investigate EBV in relation to gastric cancer in this thesis.

Genetic Factors

Up to 3% of the total gastric adenocarcinoma burden is associated with an inherited predisposition syndrome(43). Among them, inherited gastric cancers of the diffuse type are prominent within this thesis I shall term as hereditary diffuse gastric cancer

(HDGC). The identification of a germline mutation inactivating the gene encoding for E-cadherin (CDH1) in a Maori family from New Zealand was the start point to understand HDGC pathogenesis. In HDGC families, abnormal E-cadherin gene carriage is found associated to an 80% risk (Lifetime) for Gastric cancer development, leading to recommendations for genetic testing, screening, and even total gastrectomy as a prophylactic strategy in CDH1 mutation carriers(43). Other inherited predisposition syndromes include Lynch syndrome, which shows an intestinal histology in 90% of the cases, and carries a lifetime risk of around 10% for gastric cancer (of note, this risk is much higher for colorectal or endometrial cancer); ovarian and breast cancer hereditary syndrome because of germline mutations of BRCA1 and BRCA2; p53 mutations that preceeded Li-Fraumeni syndrome; and the much rarer familial adenomatous and juvenile polyposis syndromes which are associated with the hererozygotic carriage of APC gene germline mutations, BMPR1A and SMAD4 genes, and STK11 gene, respectively(44). The relationship with the BMP pathway and gastric cancer is intriguing. BMP pathway activity and Hedgehog pathway are closely linked, and in contrast to the BMP pathway, exaggerated Hedgehog pathway activity is subject to treatment by FDA and EMA-approved pharmacological inhibitors. This is one of the factors (see also below) that drove me in the course of this research to explore the potential of the Hedgehog pathway in gastric carcinogenesis.

Other Miscellaneous Factors

Pernicious anemia can result from an autoimmune disorder characterized by atrophic damage restricted to the gastric body mucosa (gastric atrophy type A). This condition confers significant risk for gastric cancer development, with a similar incidence rate as seen in gastric atrophy caused by *H. pylori* (gastric atrophy type B)(45). The associated risk appears *H. pylori* infection independent, although the potential interaction between infection and pernicious anemia has not yet been thoroughly studied. Prior gastric surgery for benign disorders (mainly gastric ulcer) was an established risk factor for adenocarcinoma development before the discovery of *H. pylori*. It is not clear if a prior gastric surgery is itself associated gastric cancer risk factor in the remnant stomach (*e.g.,* acting synergically with *H. pylori* through mechanical adverse effects of the surgery, i.e., bile reflux) or if it is merely a surrogate for long-term infection with *H. pylori* with the more aggressive CagA positive strains(46, 47). Ionizing radiation has been shown to provoke risk for cancer development, including gastric carcinoma(40). The best evidence comes from the an

atomic-bomb survivor longitudinal study involving 38,576 Nagasaki and Hiroshima citizens in Japan that were followed up between 1980 and 1999. Increased risk for gastric cancer in persons professionally exposed to ionizing radiation, e.g., astronauts has not been established. In contrast, in this thesis, I shall also reflect on the potential of space travel to convey lessons for better treatment of gastric disease.

The Hedgehog Signalling

Hedgehogs constitute a family of morphogens of pivotal importance for gestation in general and stomach development in particular. Intriguingly, gastric Hedgehog signaling remains active in the adult phase of life and where it is responsible, amongst other functions, for maintaining gastric pit-gland asymmetry. In this sense, the stomach is a beautiful example as to illustrate how morphogen signaling contributes to morphostasis in adults.

The importance of Hedgehog signaling in many forms of cancer in conjunction with its link to gastric developmental processes has prompted a substantial research effort investigating the potential usefulness of targeting Hedgehog signaling in gastric oncogenesis. As a result, in gastrin-mediated compartment expansion and viral and Helicobacter-dependent gastric carcinogenesis, the role of Hedgehog signaling is now relatively well understood. Furthermore, when the malignant disease is established, the evidence indicates that hedgehog signaling may provoke drug resistance. Now that the hedgehog inhibitor Vismodegib has come available, which has proved useful in other kinds of cancer (especially basal cell carcinoma), it is tempting to propose clinical trials using this compound for patients who have gastric cancer and accordingly various of such studies have now been initiated. In this thesis, I aim to comprehensively investigate this angle to explore the potential of targeting Hedgehog in preventing and combating gastric carcinogenesis.

Role of Hedgehog Signalling in Gastric Homeostasis

Ever since its initial detection in Drosophila, Hedgehog is associated with foregut development. The mammalian genome expresses three (3) Hedgehog ligands. In the mucosa of the embryonic foregut, Sonic Hedgehog is profoundly present[52]. All over the gut but especially in foregut-derived organs such as the lung, Sonic Hedgehog is expressed both in embryogenesis as well as in adults [53-56], while in the small intestine adjacent to the stomach expression is profoundly less marked (see

Table.1)[52, 57]. Functionally, Sonic Hedgehog controls maturation and differentiation of epithelial cells in the adult stomach[57-59].

During the progression from the inflamed stomach to gastric cancer, an important step is the loss of acid-production by the parietal cells followed by replacement of these parietal cells by mucus-secreting cells that express spasmolytic polypeptide (SP) or trefoil factor 2.[60]. This process is named, especially in mice but also in human subjects, SP-expressing mucosa (SPEM) and this is a type of oxyntic gland atrophy[61, 62]. Together with the atrophy of parietal cells in SPEM[56]. Sonic Hedgehog expression diminishes in parietal cells as well[63, 64]. The expanding SPEM compartment that replaces the parietal cell compartment also produces Sonic Hedgehog, but this remains inactive as the SPEM compartment fails to convert the unprocessed full-length 45-kilodalton preform of Sonic Hedgehog to its cleaved 19 kD mature form that is capable of eliciting morphogenetic signaling.[65][59, 63] ,[64, 66].

Recent insight as to why gastritis may reduce processing of Sonic Hedgehog to its active form has been gained and has been linked to the absence of gastric acid under atrophic conditions[67, 68]. The hypochlorhydria associated with atrophy of parietal and zymogenic (chief cell) lineages affects the production of the zymogens. Especially reduced serum levels of pepsinogen I (or pepsinogen A) (when compared to pepsinogen II (or pepsinogen C)) are noted in patients with atrophic gastritis [69-75] and assessment of the levels of the two zymogens in serum is clinically useful for indicating pre-neoplastic changes in the stomach[75]. Pepsinogen A is produced primarily in the mouse corpus by parietal cells, whereas pepsinogen C produced by both mucous neck and chief cells throughout the stomach, which may account for the differential sensitivity of the two pepsinogens for gastric atrophy[67]. In the stomach, upon acidification Pepsinogens A and C are converted to the enzymatically active aspartic proteinases, pepsin A and pepsin C, through intramolecular self-cleavage[75, 76]. Pepsin A prefers to cleave proteins at hydrophobic and aromatic amino acid residues, particularly at phenylalanine (F), when the pH is less than two. By contrast, pepsin C act on a broader range of substrate peptides and is less pH sensitive when compared to pepsin A[76, 77]. Experiments employing site-directed mutagenesis show that pepsin A cleaves the nascent 45-kilodalton Sonic Hedgehog polypeptide at residue 200 (SGGCF200|P) to generate the active 19-kilodalton form, whereas pepsin C does not cleave Sonic Hedgehog[67]. This provides a mechanistic explanation as to why SPEM is associated with reduced Sonic Hedgehog signaling.

The relationship between Sonic Hedgehog and both physiology and pathophysiology call for further studies investigating the usefulness of targeting Sonic Hedgehog signaling for combating gastric cancer and delineating the mechanistic details of its signaling as to obtain better insight in the fundamental mechanisms underlying the action of Sonic Hedgehog.

Regulation of Gastrin and Gastric Acidity by Hedgehog ligands

This notion is further supported by studies examining the impact of Hedgehog signaling on gastric physiology in vivo. An example is a transgenic mouse that secretes an endogenous inhibitor of Hedgehog ligands called Hedgehog-IP under control of the H^+, K^+-ATPase β subunit promoter, which is specific for parietal cells(48). The results show reduced secretion of gastric acid by the parietal cells. Hypochlorhydria, in general, stimulates gastrin gene expression through a decrease in somatostatin production by the enterochromaffin-like cells of the stomach (49). Accordingly, the Hedgehog-IP model displays increased plasma gastrin with concurrent decreased somatostatin production. Hence the loss of Hedgehog signaling is sufficient to activate the standard feedback mechanisms associated with loss of parietal cells that are typically attributed to gastrin and somatostatin and are linked to oncological transformation in the stomach(50). Furthermore, both antral G and D cells possess primary cilia, organelles protruding from the plasma membrane, which are intimately linked with transduction of Hedgehog signals and it is thus likely that these cells are subject to Hedgehog effects(51, 52). In apparent agreement, transgenic overexpression of GLI2, a transcription factor active in canonical Hedgehog signaling, suppresses gastrin gene expression(53). It would thus be interesting to study the cilium-specific effects of Hedgehog signaling (*i.e.,* those effects of Hedgehog that do not involve the first Hedgehog receptor Patched but do involve the second Hedgehog receptor Smoothened –see later this thesis) as these may provide further insight into the link between Hedgehog and gastric physiology. In the present thesis, I aim to do so, albeit in an artificial model system.

Cross-Links between Hedgehog Signaling, Chronic Inflammation leading to Gastric Cancer

The further imperative for studying Hedgehog signaling in the context of this thesis comes from the significant role of this morphogen not only in gastric development and homeostasis but also from its role in neoplastic transformation(54). Although

the extensive literature on the action of Sonic Hedgehog proteins and their downstream targets exists, the body of contemporary biomedical literature has not yet been systematically analyzed about the role of the morphogen in gastric pathophysiology. Nevertheless, it is clear that Sonic Hedgehog is highly expressed in gastric cancer cell lines(55). Although increased levels of Sonic Hedgehog have been reported in gastric cancers, its specific role in gastric transformation appears elusive but carries significance because of the availability of Hedgehog antagonists.

In this context is important to note that the phenotype of infiltrating myeloid cells in inflammatory reactions changes over time as to generate MDSCs (myeloid-derived suppressor cells). The latter are a heterogeneous group of immune cells from the myeloid lineage that possesses strong immunosuppressive activities rather than immuno stimulatory properties. Although their mechanisms of action are not clear yet, it is clear that cancer tissues with high infiltration of MDSCs are associated with poor patient prognosis and resistance to therapies. Importantly MDSC generation requires Hedgehog signaling. Expression of GLI1 (a pivotal transcription factor in canonical Hedgehog signaling), is an early indicator that the myeloid cells recruited during chronic inflammation are transforming towards a MDSC phenotype in the stomach(56-59). The ability to track these cell types in the pre-neoplastic state broadens options for the more efficient screening of subjects predisposed to develop gastric cancer eventually as well as to expand opportunities for prophylactic therapy once atrophic gastritis develops, including antagonists of mTOR (mechanistic antagonist of rapamycin)(60-62). Although Hedgehog antagonists been used for other cancer types, their use in clinical trials for gastric cancer is still in its infancy (63). Where initiated, those tests appear to focus on targeting CD44-positive gastric stem cells to treat metastatic disease(64). In this thesis, I shall systematically address the potential promise of such work.

Immune checkpoint blockade and Gastric Cancer

MDSC may well have a negative influence on the course of gastric cancer by impairing immune responses to the oncological process. But also aberrant activation of other immune controlling mechanisms may well have important negative contributions here. Such mechanisms are now often called “checkpoints” which refers to a broad spectrum of either co-receptors or ligands that are widely expressed by immune cells. Importantly, such checkpoints regulate immune cell activation. The “inhibitory checkpoints” represent those molecules that play an important role

in preventing over-activation of the immune system and are important to maintaining self-tolerance. In this way, potentially deleterious immune attack by the host immune system can be constrained, but it is clear that at least some cancers pervert this system to escape immunosurveillance. It is well possible that in the context of the gastric cancer cell immune microenvironment, co-inhibitory receptors may pose a threat to the host's health by preventing an immune response against gastric cancer. Both co-inhibitory receptors and ligands are highly expressed in a large number of malignancies. This high expression allows for successful evasion of antitumor immune responses. One of the most promising tumor immunotherapy strategies is to interrupt these immune "brakes" by blocking antibodies that prevent interactions between receptors and their cognate ligands. As shown by recent clinical trials, targeting either the PD-1/PD-L1 or CTLA-4 pathways have yielded exciting results. However, there remains insufficient study and understanding of specifically gastric cancer in this respect, especially when compared to other malignancies such as melanoma or lung cancer. This consideration has prompted me to explore this aspect of the gastric cancer process as well.

The scope of this thesis

The pivotal role of *H. pylori* in progression to gastric cancer calls for better understanding of the relation between its genetic make-up and such progression but also a comparison of diagnostic strategies in countries with a relatively high and low incidence of infection with the bacterium. As stated above, the importance of Hedgehog signaling in many forms of cancer and especially in conjunction with its link to gastric cancer developmental processes, has prompted a substantial research effort investigating the potential usefulness of targeting Hedgehog signaling in gastric oncogenesis. As a result, in gastrin-mediated compartment expansion, viral and Helicobacter-dependent gastric carcinogenesis, the role of Hedgehog signaling is now relatively well understood. Furthermore, when the malignant disease is established, the evidence indicates that hedgehog signaling may provoke drug resistance. Now that the hedgehog inhibitor Vismodegib has come available, which has proved useful in other kinds of cancer (especially basal cell carcinoma), it is tempting to propose clinical trials using this compound for patients who have gastric cancer and accordingly various of such studies have now been initiated. As such, this thesis seeks to summarize clinical experiences and outcomes in the ongoing clinical trials of Hedgehog inhibitors, but I also try to obtain insight into the fundamental aspects of signaling by Hedgehog ligands. A similar situation holds

true concerning immune checkpoints inhibitors in Gastric cancer. Thus I study the use of anti-PD-1, anti-PD-L1, and anti-CTLA- 4 monoclonal antibodies in gastric cancer. In addition, I aim to link the genetic makeup of *H. pylori* infection to disease progression. Finally and speculatively I also aim to investigate the potential usefulness of unconventional model systems for a better understanding of the mechanisms potentially constraining immune activity, which may further boost effectivity of such immunocheckpoint-targeting drugs. The results of these efforts will be integrated into a final discussion as well. In conjunction I hope this thesis will foster new thinking on the management of gastric cancer.

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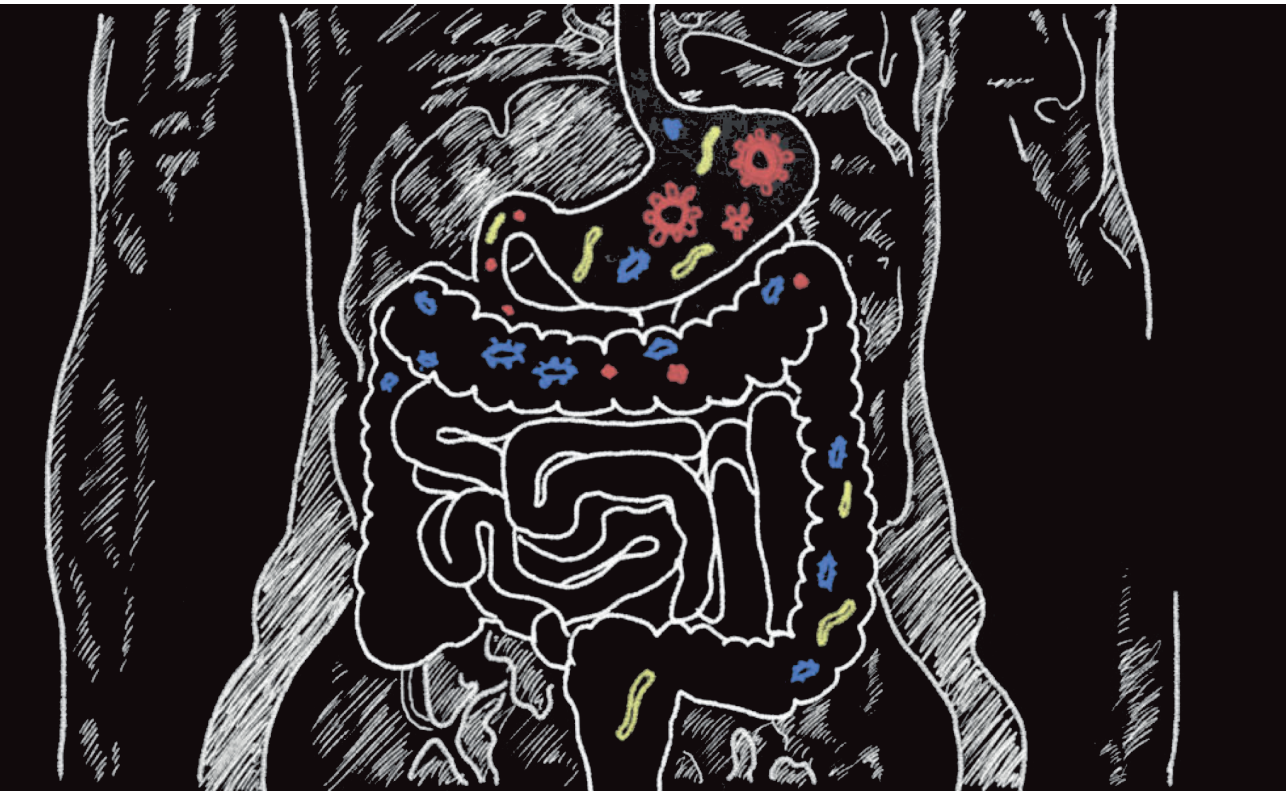

Chapter 2

Gastric cancer and the Hedgehog Signalling Pathway: Emerging New Paradigms

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Abstract

Ever since its initial discovery in Drosophila, hedgehog signaling has been linked to foregut development, The mammalian genome expresses three Hedgehog paralogues, sonic hedgehog (Shh), Indian Hedgehog, and desert hedgehog. In the mucosa of the embryonic and adult foregut, Shh expression is the highest. It has now become clear that hedgehog signaling is of pivotal importance in gastric homeostasis. Aberrant activation of hedgehog signaling is associated with a range of pathological consequences including various cancers. Also in gastric cancer, clinical and preclinical data support a role of Hedgehog signaling in neoplastic transformation, and gastrointestinal cancer development, also through cancer stroma interaction. Technological advance are facilitating monitoring Hedgehog signaling broadening options for the more efficient screening of individuals predisposed to eventually developing gastric cancer and targeting Hedgehog signaling may provide opportunities for prophylactic therapy once atrophic gastritis develops. Nevertheless, convincing evidence that Hedgehog antagonists are of clinically useful in the context of gastric cancer is still conspicuously lacking. Here we analyze review the role of Hedgehog in gastric physiology and the potential usefulness of targeting Hedgehog signaling in gastric cancer.

Mini-abstract

Hedgehog signaling has a pivotal role in gastric development and homeostasis but also appears to associated with neoplastic transformation, and further development towards invasive carcinoma in the stomach. Thus monitoring Hedgehog signaling may provide an opportunity for more efficient screening of subjects predisposed to developing gastric cancer. Targeting Hedgehog signaling may be useful for prophylactic therapy once atrophic gastritis develops. Although Hedgehog antagonists have been used for other cancer types, data documenting its clinical usefulness in gastric are conspicuously lacking, and studies aimed at providing definitive answers here are called for.

MeSH keywords

Patched, Receptors; Smoothened Receptor; Zinc Finger Protein GLI1, Precision Medicine

Introduction

Hedgehog proteins are fundamental regulators of embryological development, and tissue homeostasis in adult organisms. Disturbed hedgehog signaling is associated, amongst others, with a range of congenital disabilities, oncological malignancies and immunological defects[1]. Hedgehog proteins intercellular signaling molecules of unusual and fundamental relevance as also illustrated by their substantial conservation across the animal kingdom[2-5]. Initially recognized as a segment polarity gene in Drosophila, now numerous vertebrate paralogues have been found, and in mammals, these include Sonic Hedgehog (Shh), Desert Hedgehog (Dhh), and Indian Hedgehog (Ihh), with Shh being the most comprehensively characterized[5]. Although mainly associated with organogenesis and general and embryological formation of the intestines, in particular, Hedgehog signaling remains active until death, and serves to maintain lifelong histostasis in the intestinal tract and also the immune system[6-8]. The pathophysiological importance of Hedgehog signalling is illustrated by the observation that continuous hedgehog signalling is an essential permissive factor in endodermal cancer development[9-11]. With regard to the above, especially the stomach is relevant, where the morphogen not only maintains pit-gland asymmetry, but also fosters the development of gastric cancer, homeostasis, and neoplastic transformation[12-14]. Part of this nefarious functionality is related in the initiation of gastric inflammation due to Helicobacter infection[12]. As stated, although classically associated with gestation, the role of Hedgehog pathway has also important functionality beyond embryogenesis and a potentially vicious one with respect to oncological disease. In cancer, both autocrine Hedgehog signalling and paracrine signalling (through the tumor stroma that would thus nurture the tumour cells) of Hedgehog ligands is well-established[15, 16]. Both autocrine and paracrine Hedgehog signalling should be sensitive to pharmacological inhibitors and are thus tested in clinical trials in addition to an intense preclinical research effort[15, 17]. The importance of Hedgehog signalling gastric pathophysiology has led to hopes that pharmacological inhibitors of this signalling may become useful for combating oncological disease in the stomach and this consideration prompted us to review here the detailed molecular mechanism by which Hedgehog influences gastric pathophysiology and to evaluate the evidence that anti-Hedgehog strategies will prove effective in this respect.

The physiological importance of Hedgehog signaling in the physiology of the proximal tract is illustrated by the phenotypes observed in mice with genetic loss of

Hedgehog paralogues. Genetic knockout of both Shh and Dhh provoke by malrotation of the gastrointestinal tract, oesophageal atresia, gastric overgrowth and other gross abnormalities[18-20]. The specific importance of Hedgehog signalling for the stomach in this respect is illustrated by the observation in mice from embryonic day 16 onwards as dichotomy occurs in that the foregut and at the level of antrum and pyloric border region which becomes dramatically more active with respect to Hedgehog signalling as compared to the adjacent duodenal tissues[21], and also is proposed to maintain pit-gland asymmetry in the stomach[7, 22]. Thus the relevance of Hedgehog signaling for gastric physiology seems evident. With regard to pathophysiology, Hedgehog signaling is suggested to be pivotal for gastric cancer progression in both of humans and animals, but a definite etiological role has not yet been shown for this pathway in gastric cancer. To further analyze the precise evidence available in this respect it is essential first to review the molecular details of the molecular signaling involved[23].

Hedgehog signaling: An overview

Hedgehog signaling in general is unusual and complicated, and an immense scientific effort has been necessary to unravel its general principles[15, 24-26]. Signaling is initiated by the different Hedgehog ligands, in casu Shh, Ihh and Dhh. In the classical Hedgehog signal pathway activation, these different ligands bind a common cognate membrane-bound receptor called Patched that has approximately 1,500 amino acids. The protein transverses the plasma membrane twelve times and thus strongly resembles abc transporter proteins. In accordance both the The N-terminal and C-terminal domains of the protein reside at cytoplasmic side of membrane, The tertiary conformational of Patched allows Hedgehog ligands to bind via the interaction with two extracellular loops[15, 27]. There are two genes encoding Patched receptors in humans; which are dominated as PTCH1 and PTCH2, and differ slightly with respect to their amino acid configuration in the N-terminal region[15, 27]. While both PTCH1 and PTCH2 receptors are associated with numerous human cancers, with respect to gastric cancer especially PTCH1 is the relevant gene product. The function of Patched is to exclude a second receptor, called Smoothed from the primary cilium and retain Smoothened in a vascular compartment/ Binding of Hedgehog to PTCH release this inhibition enabling further downstream signalling[15, 28]. Figure 1 provides a graphical representation.

Following binding of Hedgehog to Patched. Smo translocates to the the primary cilium in the cell membrane. The subsequent signalling culminates in altered transcription through Gli transcription factors. The molecular details of Smoothened signalling to Gli is still partly obscure but involves the microtubule transport proteins[15, 29]. The Gli family are members of the Kruppel family of zinc finger transcription factors and a role for three different Gli proteins in Hedgehog signalling has been identified, Gli1, Gli2, and Gli3, each with a distinctive role[15], In the absence of Smoothened activation, GLI1 and GLI2 are transcriptional repressors, but following activation of the pathway these proteins are converted to transcriptional activators[15, 30]. The role Gli3 appears to be mainly as a negative regulator of Hedgehog signalling. It is thus possible to interfere with Hedgehog signalling at different levels, although clinically the use of Smoothened inhibitors has gained most attention.

Role of Hedgehog Signalling in Gastric Homeostasis

Ever since its initial detection in Drosophila, Hedgehog has long been associated with foregut development. Of the three mammalian Hedgehogs (3) Shh levels are most highly expressed h, in the mucosa of the embryonic foregut[31]. Also in other foregut-derived organs such as the lung, Shh expression is prominent, reflecting the embryonal situation[32-35]. Table 1 lists expression patterns in physiology and pathology. Interestingly high Shh expression in the stomach is lost upon development of intestinal metaplasia (Table.1), suggesting that gastric epithelium-specific effects of the morphogen[12, 36]. Indeed Shh controls gastric epithelial cell maturation and differentiation in the adult stomach[7, 36, 37].

During progression from the inflamed stomach to gastric cancer the epithelium goed through defined series of morphological transitions. First, the acid-producing parietal cells are lost and are replaced by mucus-secreting cells that express spasmolytic polypeptide (SP) or trefoil factor 2.7 [38]. Mostly in mice, but also in human subjects, the presence of SP-expressing mucosa (SPEM) defines gland atrophy[39, 40]. Together with atrophy of the parietal cells[35] Shh expression diminishes[41, 42] Although Shh expression diminishes along with the loss of parietal cells [16] the expanding mucous cell compartment or SPEM continues to produce Shh in both human subjects [37, 41] and rodents[42, 43] but appears to remain as the unprocessed pre-morphogen. Thus functionally expression is lost. Studies suggest that aberrant Hh signaling in cancer functions mainly as either

autocrine or paracrine regulator. Especially in stem cell niche Processing of Shh to its active form (19 kilodaltons) in parietal cells becomes compromised in the absence of gastric acid [44, 45] Atrophy of parietal and zymogenic (chief cell) lineages result in hypochlorhydria and reduced serum pepsinogen I (A) levels compared to pepsinogen II (C)[46-52]. These zymogens are proteins encoded by different gene loci that are used clinically to identify pre-neoplastic changes in the stomach[52]. Pepsinogens A and C are converted to the enzymatically active aspartic proteinases, pepsin A and pepsin C, through intramolecular self-cleavage[52, 53]. Pepsinogen A is produced primarily in the mouse corpus by parietal cells, whereas pepsinogen C is mainly produced by both mucous neck and chief cells throughout the stomach[44]. This result is consistent with the exclusive expression of pepsinogen A in the human corpus and not the antrum, whereas pepsinogen C marks mucous cells of both the antrum and corpus (www.proteinatlas.org). Pepsin A prefers to cleave proteins at hydrophobic and aromatic residues, particularly at phenylalanine (F) when the pH is less than 2. By contrast, pepsin C recognizes a broader consensus site and uses more comprehensive pH spectrum than pepsin A[53, 54]. Explicitly, it's shown using site-directed mutagenesis that pepsin A cleaves the nascent 45-kilodalton Shh polypeptide at residue 200 (SGGCF200|P) to generate the active 19-kilodalton form, whereas pepsin C does not cleave SHH peptide[44]. This may account for absence of Shh expression in atrohic gastritis.

Regulation of Gastrin and Gastric Acidity by Shh

Several studies have examined the impact of blocking Hedgehog signalling in vivo, for instance by employing a transgenic mouse that secretes a natural -inhibitor of Hedgehogs called HHIP employing the parietal cell-specific H+, K+-ATPase β subunit promotor[65]. This approach showed that loss of Hedgehog signalling in parietal cells reduces H+, K+-ATPase gene expression and gastric acid secretion[65]. Usually, hypochlorhydria stimulates gastrin gene expression through a decrease in Somatostatin levels[66] Accordingly, increased plasma gastrin occurred in the HHIP transgenic mice, concomitant with reduced somatostatin expression. Both antral G and D cells possess primary cilia, organelles protruding from the plasma membrane, essential for transducing Hedgehog signalling[67, 68]. Therefore, gastric endocrine cells may well be capable of responding directly to Shh. Functionally, this idea is supported by the observation that that transgenic overexpression of GLI2 suppresses gastrin gene expression[69]. Taken together, the

production of Shh by parietal cells and the ability of gastric endocrine cells to sense the ligand through primary cilia are consistent with a central role for Hedgehog signaling in the feedback regulation of gastric acidity

Modes of hedgehog signaling in gastric cancer

Upregulation of Hedgehog signaling pathway is involved in tumour development[36]. De Sauvage and Rubin postulated models for Hedgehog signalling in human cancer development[36]. The type I cancers are ligand-independent and involve constitutive stimulation of downstream signalling molecules (e,g, loss of Patched) and an example is basal cell carcinoma. Type II are cancers ligand-dependent were both the autocrine, or juxtacrine signaling mechanisms are involved as seen in pancreatic tumors. In type III cancers also ligand-dependency is observed but this type displays paracrine type signalling[36]. Table 1 provides information on these type of tumours in the context of stomach cancer. Remarkably, these models ignore the involvement of non-canonical signalling mechanisms. A number of studies have evaluated the role of cyclin B1 interaction with Patched, in which a Ptch1-cyclin B1 complex is formed at the plasma membrane in a cyclin kinase-1 (Cdk1)-dependent fashion[56, 57]. This results in a reduction in the mitotic index by the separation of cyclin B1/Cdk1 complex from the nuclear machinery resulting in decreased proliferation. Shh binding to patched release the complex and thus fosters cell cycle progression through G2/M phase checkpoint. Obviously Smoothened inhibitors do not affect this process. Another study documents Hedgehog-independent activation of Patched through the action of proteases and in particular Caspase 3, splitting the C-terminal from Patched[36, 58, 59]. It islikely that such non-canonical signalling

Table 1: Small molecules related Hedgehog expression in Gastric Cancer.

S/N	Homologs	Normal stomach	Stomach Metaplasia	Tumour		Techniques	Ref
				Intestinal	Diffuse		
1	Shh	Gland Epithelium	Undetectable	elevated	Undetectable	IHC,RT-PCR IHC, RT-PCR RT-PCR,IHC,IMF	(65) (66) (67) (68)
2	Ihh	Pit Epithelium	Undetectable	elevated	Undetectable	IHC, RT-PCR PCR,IHC,IMF	(65) (55)
3	Dhh	Gland Epithelium	Undetectable	elevated	elevated	IHC, RT-PCR PCR,IHC,IMF	(65) (55)
4	Ptch1	Pit Mesenchyme	Detected	elevated	elevated	IHC, RT-PCR RT-PCR, IMF RT-PCR, LacZR	(65) (55) (8)
5	SMO	Pit / Gland Mesenchyme	Detected	elevated	elevated	IHC, RT-PCR PCR,IHC,IMF	(65) (55)
6	Gli 1	Pit / Gland Mesenchyme	Undetectable	elevated	elevated	IHC, RT-PCR PCR,IHC,IMF	(65) (55)
7	Gli 2	Pit Mesenchyme	Undetectable	elevated	elevated	IHC, RT-PCR PCR,IMF	(65) (55)
8	Hip	Detected	Not Reported	Undetectable	Not Reported	RT-PCR, IMF	(55, 65)
9	BOC	Pit	Undetectable	elevated	elevated	IHC, RT-PCR	(65)

NOTE: NA**:** not available; Hh: Hedgehog; Gli: glioma-associated oncogene; Pitch: Patched; Smo: Smoothened; Shh: Sonic Hh; IHC = immunohistochemistry, ISH= in situ hybridization, LacZ reporter. RT-PCR real-time PCR.

Cross-Links between Hedgehog Signaling, Chronic Inflammation and Gastric Cancer

As stated, Hedgehog signalling in the stomach plays a significant role in gastric development, homeostasis, and neoplastic transformation[70]. Initially Shh was somewhat ignored in the context of gastric cancer, despite the evidence that Shh is highly expressed in gastric cancer cell lines[64]. Although increased levels of Shh have been reported in gastric cancers, its specific role in gastric transformation remains elusive but carries significance because of the availability of Hh antagonists. A link exists through the immune system; several studies show that in gastritis the phenotype of infiltrating myeloid cells changes over time to become myeloid-derived suppressor cells (MDSCs) and that this phenotypic switch requires Hedgehog signaling. More specifically, expression of GLI1, which targets Slfn4 (mice) and SLFN12L and SLFN5 (humans), is an early marker for chronic inflammation-associated myeloid cells in their transition towards the MDSC phenotype. As MDSCs are important for immune-evasion for transformed cells, Hedgehog signaling can thus favor neoplastic development.

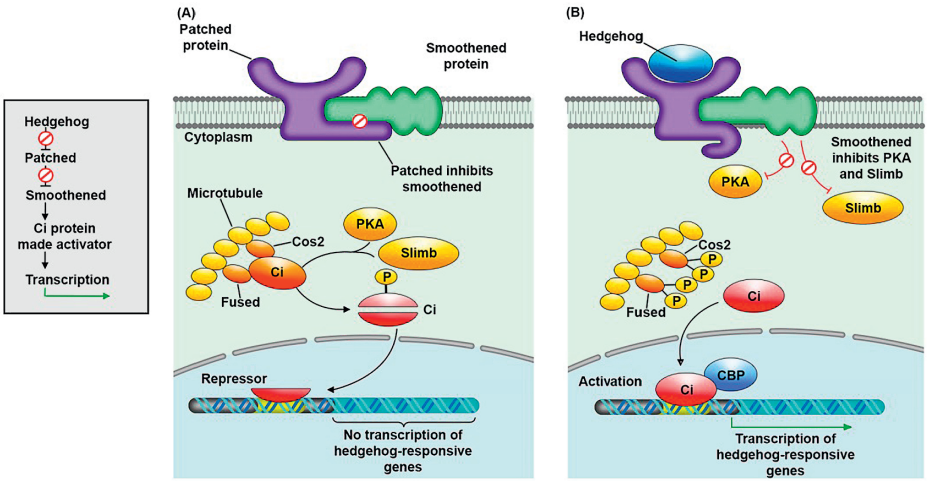

Figure 1. A simplified model of the mammalian Hh pathway. In the 'OFF' state, Patch inhibits the activity of Smo. Inactive Smo is unable to inhibit Sufu, which promotes processing of the Gli transcription factors in favor of shorter, transcriptional repressor forms (GliR). In the 'ON' state, Hh ligands bind to and inhibit Patch, thus releasing Smo activity and in turn blocking Sufu. Gli processing is then shut down, leading to the accumulation of transcriptional activator forms (GlIA).

Hedgehog Signaling pathway inhibitors

The Hedgehog signaling pathway is a significant target in cancer therapy. Various molecules that may inhibit the pathway have been evaluated in both preclinical and clinical studies. These inhibitors include: SMO inhibitors, ligand-receptor inhibitors, Gli targeted inhibitors, and these classes of molecules are adstructed in figure 2.

SMO Inhibitors

Cyclopamine, originally isolated from the flower veratrum californicum that causes birth defects when eaten by pregnant cattle, was the first compound that was used as a Hedgehog inhibitor and it targets SMO. Consequently, GLIs activation is inhibited. In the clinic, it is side effect-prone and exhibits substantial toxicity[79]. Frustratingly, mice studies involving rhabdomyosarcoma and osteosarcoma models, reveal no significant impact on cancer cells metastasis or growth[80, 81]. From mouse model studies, skin ulcerations and scrubby coat were reported as particularly noticeable skin toxicity; halted even studies initiated to ascertain cyclopamine therapeutic dosing[81]. Adverse effects of cyclopamine in conjunction with other limitations such as acid sensitivity and poor solubility have now halted its clinical development as a potential compound for the treatment of cancer and prompted efforts aimed at. Identifying molecules potentially more suited. This led to the development of the acid stable and water-soluble compound vismodegib (GDC-0449), which was eventually approved by the FDA for the treatment of advanced (Locally), recurrent and metastatic skin cancer. Another semisynthetic novel analogue of cyclopamine (Saridegib (IPI-926) was developed with enhanced potency and metabolically stable[82]. Other inhibitors that impede SMO include LEQ506, PF-0449913, LDE-225 (erismodegib), and BMS-833923. It is important to note that such compounds will not impair Patched-dependent Smoothened-independent non-canonical signaling**.**

GLI inhibitors

Also in view of resistance development against vismodegib through SMO mutation efforts have been made to target Gli. Two inhibitory compounds (GANT 58. And GANT 56) were identified through cellular screens aimed at identifying compounds

able to inhibit transcription mediated by GLI. Both compounds at the cellular level have been revealed. Understanding the mechanism of action of these compounds at a molecular level is still incomplete. GLI1 posttranslational modification by GANT 61 impedes binding to DNA or changes the conformational structure of the GLI1-DNA complex. In xenograft mice model of human prostate cancer cells inhibition of cancer cell growth is observed[83]. A study by Hyman et al. also succeeded in identifying four further Hedgehog inhibitors apparently acting downstream of Smoothened: HPI-1, HP-2, and HP-3 are thought to inhibit signaling by targeting a posttranslational modification of GLI or interaction between GLI and a co-factor. HPI-4 was thought to be the only agent that acts by perturbing ciliogenesis, although the mechanism by which HPI-4 disrupts ciliogenesis was not clarified[84]. Again, it is important to note that such compounds will not impair Patched-dependent Smoothened-independent non-canonical signaling.

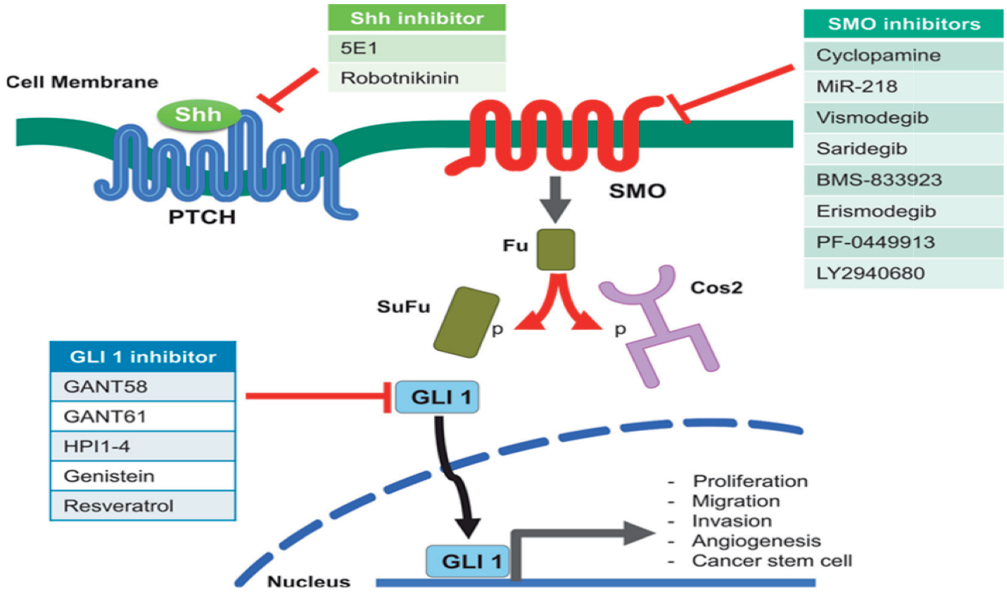

Figure 2. Molecular sites targeted by Hh signaling pathway inhibitors. These inhibitors target different components of Hh signaling, including Shh, Smo, and GLI1. These encompass natural compounds, their chemical derivatives, a monoclonal antibody, and chemicals identified from screening libraries. (Adapted from reference)(82)

A monoclonal antibody 5E1 (ch5E1) from murine-human chimeric has been proven to bind Shh and thereby to inhibit Hedgehog signaling[85]. A small molecule (Robotnikinin) was also identified as a Hedgehog signaling inhibitor in a microarray-based screening effort. Robotnikinin binds to Shh thereby inhibiting the activation of Hedgehog signaling [86]. Other new ligand processing blocking agents have been identified in high-throughput screens and appear to have different mechanisms of actions, including interfering with Shh palmitoylation by targeting Hh acyltransferase[87, 88]. These molecules have obvious potential as they also target non-canonical Hedgehog signaling.

Gastric cancer Treatment using Hh pathway targeted agents.

For gastric cancer treatment using Hh signaling targeted agents, only two clinical trials have been performed. A phase II multi-centered, randomized, a prospective clinical trial involving 124 participants was performed to determine the vismodegib efficacy, potency, and safety. For adenocarcinoma patients under FOLFOX chemotherapy, vismodegib an SMO inhibitor was administered in conjunction with this regimen. The study did not meet the primary endpoint with a no significantly-improved progression-free survival between the placebo group and vismodegib group (9.3 months vs. 11.5; $p = 0.34$), although with a noticeable tendency for prolonged progression-free survival[89]. The lack of a statistically significant result may well stem from this study being underpowered. Lack of good biomarkers that can act as surrogates for progression-free survival can also be considered a confounding factor. CD44 immunopositivity has been established as a biomarker of gastric cancer stem cells. CD44 expression was analyzed in phase II clinical trial samples of gastric tumors and were associated with improved survival. Patients who received chemotherapy alone had poor survival together with high CD44 suggesting a potential role for CD44 as a biomarker in the treatment of patients with Hedgehog signaling targeting agents[78]. In addition a BMS-833923 maximum tolerable dose phase 1 clinical trial has been performed. Capecitabine and cisplatin were used in combination with BMS-833923 in drug naïve adenocarcinoma patients. The study was completed in 2013, but the findings have not yet been reported (NCT00909402). Thus a potential role for Hedgehog inhibition in gastric is far from evident and requires more clinical testing.

Clinical Applications

Gastric cancer has long been seen as one of the most difficult gastrointestinal malignancies to treat. Encouragingly, recent progress with targeted therapies offers hope for patients with advanced gastric cancer and is substantially expanding the therapeutic armamentarium with regard to this infaust disease. As these treatments continue to be developed, we must focus on determination of predictive markers, and preferably co-develop drugs with these markers. The mechanisms underlying primary or acquired resistance to targeted agents also should be clarified to help further drug development[12]. Developing anti-Shh monoclonal antibodies as Shh antagonists is an area to explore where Hedgehog signaling pathway can be blocked at different levels[90]. Gastric cancer is a multigenic disorder influenced by *Helicobacter pylori* infection and salt intaker. Single nucleotide polymorphism (SNP) and copy number polymorphism (CNP) of genes encoding Hedgehog signaling molecules would be utilized for genetic screening of gastric cancer. Also, cDNA-PCR, microarray, and ELISA detecting aberrant Hedgehog signaling activation would be used for optional therapeutic choice. Genetic testing and precise selection of therapeutic options would contribute to the realization of personalized medicine. Several limitations account for poor treatment outcomes in gastric cancer patients amongst which include tumor heterogeneity. Due to traditional classification of gastric cancer into two categories in casu undifferentiated and differentiated types, obvious biological differences must exist. Additionally, molecular subgroups exist gastric cancer category; these include chromosomal instability tumors, stable genomically tumors, unstable microsatellite tumor and Epstein–Barr virus tumor-positive[91]. It is well possible that stratification for subtype is way forward with regard to Hedgehog inhibition for the treatment of gastric cancer.

Conclusion and future directions.

While there is good evidence that Smoothened inhibition may be useful for a selection of gastric cancers, we feel that its untargeted application on gastric cancer patients, in general, is likely to prove disappointing. In this sense efforts to select patients characterized by unusually high SMO expression in gastric tumor material with high probability to have cancers that are truly dependent on a functional Hedgehog pathway may likely yield positive results. As both approaches are

currently being attempted in clinical trials, it should prove interesting to see whether this notion holds true.

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Authors' contributions

A.I.A wrote the manuscript, and M.P.P reviewed and approved the final version.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The author has no financial or relationship conflicts to disclosure.

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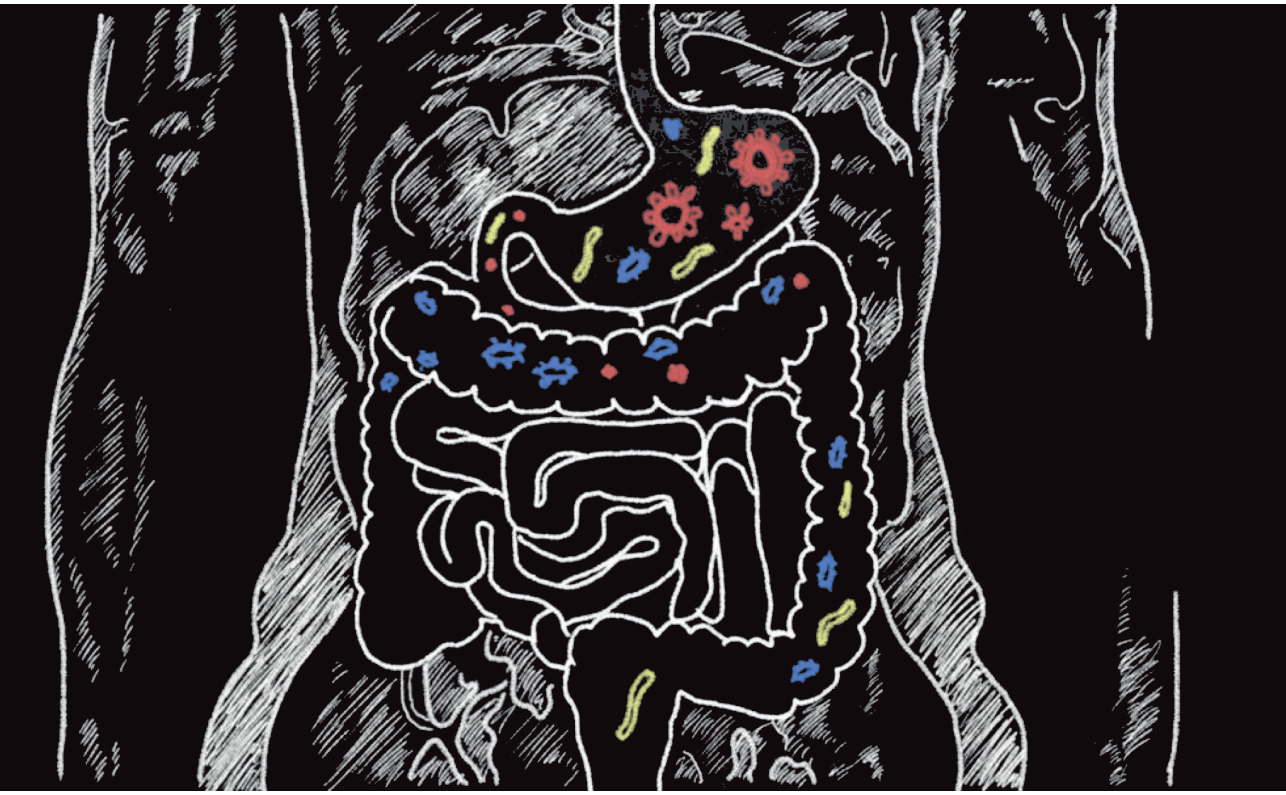

Chapter 3

Immunotherapy Checkpoint Inhibition In Gastric Cancer: A Systematic Review

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Abstract.

Objectives: Checkpoint inhibitor therapy is revolutionizing the management of the oncological disease. Despite gastric cancer being a common malignancy with a relatively poor prognosis, checkpoint inhibitor therapy has attracted relatively little attention for the treatment of this disease. We decided to perform a systematic review focusing on the potential usefulness of checkpoint inhibitors in patients with gastric cancer by reviewing all clinical trials involving checkpoint inhibitors in gastric cancer up to the 9th of September 2017.

Methods: We systematically review all randomized controlled trials on the 9th September 2017. We searched the. embase.com, Medline Ovid, Cochrane CENTRAL, Web of Science, Google Scholar, and databases of current ongoing randomized trials. A description assessment of randomized clinical trials (RCTs) was performed on Immune checkpoint inhibitors for PD1, CTLA-4 and PD-L1 (Atezolizumab, Avelumab, Durvalumab, Ipilimumab, Nivolumab, Pembrolizumab, and Tremelimumab) in gastric cancer patients.

Results: The current body of biomedical literature describes 3621 gastric cancer patients being treated with checkpoint inhibitors and compared to patients receiving chemotherapy. The PD-1 inhibitors pembrolizumab and nivolumab in conjunction appear to substantially outperform conventional chemotherapy, with more extended overall survival median (OS; 10.0 vs. 6.0 mo), a more significant objective response rate of 36% versus. 13%, p=0.10, with a reduced adverse event rate (33 % vs. 22%), although other checkpoint inhibitors appear somewhat less effective.

Translational impact: It seems that checkpoint inhibitor therapy in general, and PD-1-directed therapy, in particular, constitutes a rational therapeutic avenue for advanced gastric cancer.

MeSH keywords: Immunomodulator therapy; Gastric cancer; Biologic therapy; Biomarker

Key Points

Gastric adenocarcinoma is a very aggressive cancer with a variable response to neoadjuvant therapy and poor overall prognosis. Although neo-adjuvant chemotherapy or chemoradiation (nCRT), combined with surgery significantly improves survival of patients, novel therapeutic options against these malignancies, especially for patients refractory to standard therapies, are urgently needed.
Immune checkpoint-directed therapy is revolutionizing is oncological medicine, but its potential in gastric cancer is unclear.
PD-1-directed therapy (e.g. Nivolumab or Pembrolizumab) is associated with increased survival in gastric cancer patients refractory to other treatments and thus a therapeutic option for this group of patients. Targeting PD-L1 or CTLA4 in gastric cancer remains an unproven strategy.
In lung cancer patients likely to benefit from targeting PD-1 can be selected by staining cancer material for PD-L1. In gastric cancer, however, PD-L1 staining is not useful for selecting patients and should thus not be used for clinical decision making with respect to PD-1 inhibitors

Introduction:

Gastric cancer(GC) is a major public health problem and is the second most prominent cause of cancer-related death globally[1]. The incidence of GC, however, dramatically varies across different geographical areas, it being the highest in Japan, China, other Far Eastern countries, Russia, Middle Eastern area, and in the Pacific coast of the South American continent, whereas it is the lowest in Central Africa. Europe and North American regions share an intermediate-to-high incidence. Although only 25–30% of GC patients survive five years from the initial diagnosis, heterogeneity in survival rates is substantially and may relate to biological and genomic differences between Asian and Western populations[2]. The depressingly high mortality calls for better clinical management.

Currently, chemotherapy remains the mainstay in the treatment of advanced disease and involves a plethora of cytotoxic agents comprising platinum agents, fluoropyrimidines, anthracyclines, irinotecan and taxanes. At present combinatory chemotherapy regimens is the preferred first-line option as they result in better survival and response rates when matched to therapy involving single agents[3]. Nevertheless, it is fair to say outcomes are disappointing and alternatives are vigorously pursued. Strikingly, however, immunotherapy has been getting relatively little exposure in this respect.

Generally speaking, immunotherapy and especially immune checkpoint-directed therapy are now revolutionizing the management of oncological disease (indeed in 2013 the American Academy of science announced immunotherapy as the breakthrough of the year[4-6]). Cancers are antigenic and provoke immunity, but manage to escape their resulting destruction via a variety of mechanisms but also through activation of so-called checkpoints: inhibitory elements to limit self-damaging autoimmunity. The most prominent of this immune checkpoint inhibitory antibodies are those against cytotoxic T-lymphocyte antigen 4 (CTLA-4), receptor of programmed cell death 1 (PD-1) and its cognate ligand programmed death-ligand 1 (PD-L1). Figure 1 illustrates their mode of action and their efficacy includes a wide range of solid cancers in particular melanoma[7-9], cancer of the lung[10-12] and basal cell carcinoma[13]. Thus it is tempting to assume a possible efficacy for such checkpoint inhibitors for gastric cancer as well. Here we review the available public data and conclude that especially PD-1-directed therapy (Pembrolizumab,

Nivolumab) and PD-L1-directed cancer constitutes a novel avenue for the treatment of advanced gastric cancer.

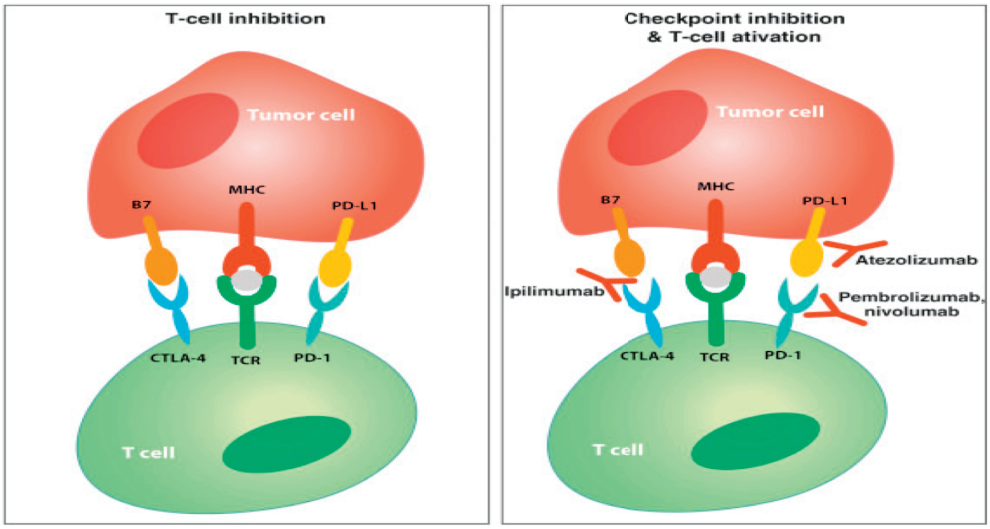

Figure 1 – Schematic depiction of T-cell co-inhibitory receptor expression and checkpoint inhibition. Antigen presenting cells (APCs) express a specific antigen that is presented to cytotoxic T cells in a major histocompatibility complex peptide and Tumor cells. The immune cell receptor recognizes presented antigens and, together with binding of costimulatory receptors (eg, CD28); this primes to lymphocytes activation and subsequently elimination of tumor cell. Interaction of co-inhibitory receptors of T cells with their corresponding ligands on APCs or tumor cells inhibits T-cell activation. Known co-inhibitory receptors are PD-1 (that act together with its ligand PD-L1) and CTLA-4. Inhibiting antibodies against these co-inhibitory receptors or their respective ligands can prevent their interaction and the subsequent inhibition of T-cell action.

Methods

Search strategy and data source

A systematic search of available articles was conducted on the 9th September 2017, using an electronic database of embase.com, Medline Ovid, Cochrane CENTRAL, Web of science, Google scholar. We looked for Extra references by exploring the reference list of carefully chosen studies was performed by a skillful librarian [W.B]. The search was done by the guiding principle of Preferred Reporting Items

for Systematic Reviews and Meta-Analyses (PRISMA) guiding principle (Fig. 2)[14]. To evaluate primary research literature on the immune therapy checkpoint in the ongoing clinical trial, we used the following search terms ("('immune checkpoint inhibitor'/de OR 'checkpoint inhibitor'/de OR 'immune checkpoint modulator'/de OR 'immune checkpoint'/de))).Immune checkpoint inhibitors for PD1, PD-L1, and CTLA-4, atezolizumab, avelumab, durvalumab, ipilimumab, nivolumab, pembrolizumab and tremelimumab''. The search was finalized by a manual screening of articles to be included in the study. Because of its unusual relevance to the subject, one single study appearing after the 9th September 2017 was included as well.

Study selection,

The population enrolled in clinical trials consisted of patients older than 18 yr who was diagnosed with the progressive phase of metastatic GC and were treated with either a mono or combined therapy of immune checkpoints inhibitors targeting either CTLA-4, PD-1, and PD-L1. Only potential prospective randomized clinical trials, phase one, two and three clinical trials were included in the review analysis. Correspondence, letters to editor, systematic and meta-analysis review, nonrandomized clinical trial, case reports, editorials and conference abstracts were excluded. If various analyses of the same clinical trial were performed, the most current or most germane article was included. Primary outcome measures included Overall survival (OS), Objective response rate (ORR) and progression-free survival (PFS), conferring to the guideline of Evaluation Criteria in Solid Tumors Response[15]. Secondary outcomes included adverse events (AEs) and efficacy of PD-L1 expression status in tissues of gastric cancer origin. Restricted searched articles which have randomized trials done in humans.

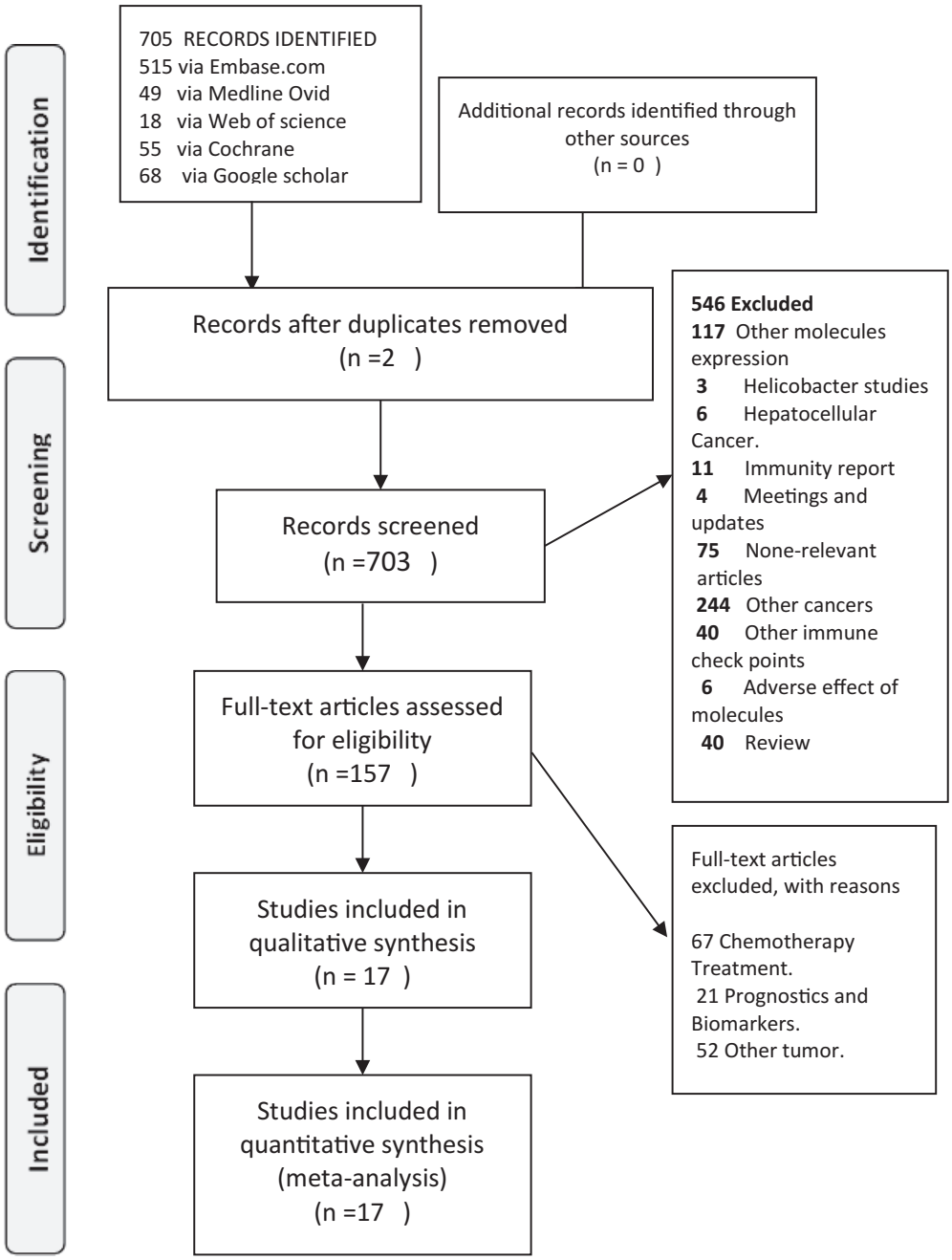

Figure 2: Schematic flowchart of selection and search strategy Flow diagram showing literature search and result selection.

Data extraction

Independent reviewers (I.A. and A.J.) evaluated relevant articles for eligibility. Moreover, discussion resolved any disagreement on included studies. The Randomized clinical trial study design, number of patients enrolled in the study, treatment regimen options, overall survival(OS), PDL-1 expression status, the follow up (media) and the OOR. Included studies by one reviewer (I.A.) and later verified by a second reviewer (A.J.) to confirm their precision and accuracy.

Data analysis.

Data were presented using descriptive analysis. Means and standard deviation were used to described continuous outcomes, interquartile range and median were used. Proportions and frequencies were also used to present specific findings. Odds ratios for response rate, HR for OSS, and PFS was stated and if they report with confidence intervals (CIs). We could not perform quantitative analysis (Meta-analysis) because of a limited number of studies.

Results.

Qualitative and systematic review analysis

The search texts identified 705 articles and an additional study published after the end date for inclusion but being of particular relevance. After removing duplicate studies, we assessed all titles and abstracts. Finally, 17publications were identified as potentially germane and were retrieved for full-text appraisal. An additional study, outside the predetermined time-interval but of general relevance was included as well. Agreeing to these inclusion benchmarks, seven randomized phase 1–3 clinical trials were carefully chosen for evidence analysis. Randomised clinical trials (5) on ten sorts of treatment, including 979 patients, all the clinical trial are multicentric and announced in English. Trials using immune checkpoints (PDL-1, CTLA-4, and PD-1). Drugs used in the clinical trial include Tremelimumab, Pembrolizumab (MK-3475), Nivolumab (MSB0010718C, Durvalumab (MEDI4736) and Atezolizumab (MPDL3280A) as seen in Table. 1. The first screening of this literature suggested that these immuno-checkpoint-targeting agents were associated with manageable levels of toxicity and potential clinical benefit in

advance gastric cancer. Hence we decided to continue with the further analysis of the available public data.

Study characteristics; Expression, Clinical Associations, and Prognosis.

Published articles including six studies on clinical trials including and 18 ongoing clinical trials with 3291 patients at the point of reference, with a most extreme example size of 451 and a base specimen size of 96 members. Most of the investigations accounted for are in Asia (Japan) and the United States, by the geographical variations in gastric cancer incidence. The first clinically tested immune checkpoint is CTLA-4, and two monoclonal antibodies (ipilimumab and tremelimumab) targeting this checkpoint have been developed for clinical use. Based on the experience in other cancers, relatively more attention is now given to the PD-1/PD-L1 checkpoint system that can be targeted by various biologicals including nivolumab, pembrolizumab for PD-1 and atezolizumab, avelumab, durvalumab for PD-L1 respectively. Although not supported by published evidence, it has already been tried to employ such medication for precision medicine, patient selected for experimental immuno-checkpoint inhibition based on high PD-L1 expression (immunohistochemistry for PD-L1 behaves relatively well, hence the choice this marker appears mainly pragmatical). For instance, a phase I trial was conducted involving a cohort of 39 patients selected for PD-L1 immunochemical positivity as assayed employing the 22C3 antibody. Patients were treated after every two weeks with 10 mg/kg of pembrolizumab. There was a 22% overall response rate, but 53.1 % experienced some decrease in tumor size, which is a good result for advanced gastric cancer. Remarkably, an impressively long median response of 40 weeks (range 20–48+) was observed resulting in 11.4 months of the overall survival rate[4]. As stated, these results are remarkable as the patient group involved is refractory to other treatments. A question that needs to be urgently addressed in this respect is the usefulness of triaging patients for PD-L1 expression[5]. The potential of targeting PD-1 in gastric cancer has recently been substantially bolstered by a phase III trial using nivolumab. This trial, which involved heavily pre-treated patients showed 26 percent twelve months survival in the Nivolumab group versus only eleven percent in the placebo group. The results of all published trails involving immune-checkpoint inhibition are provided in Table 1.These antibodies include: BMS-936559, atezolizumab (MPDL3280A),durvalumab (MEDI4736),and avelumab (MSB0010718C, which has proven efficacy in gastrointestinal malignancies[16-18]). Concerning the latter out of 346 eligible patients enrolled,

only 16 patients had gastric cancer with overall response rate outcome of 25%[21] (Table 1). Other studies are ongoing (table 2). Thus confidence in results will be boosted. Intriguingly, anti-CTLA4 strategies appear much less effective as compared to anti-PD1/PD-L1 strategies and it would be interesting to explore whether is a reflection of the fundamental biological immunology of advanced gastric cancer. Despite a lack of fundamental understanding as to the specific sensitivity of the gastric cancer to PD1-PD-L1 checkpoint inhibition, it is fair to say that It molecules targeting the latter system appear a promising novel avenue for the treatment of advanced gastric cancer. We shall discuss the various trials below.

Table 1: Main features of included clinical trials with immune checkpoint therapies in gastric cancer

Study /References	Target	Drug	Phase	Tumor Type	Number of enrolled patients	Clinical End Point
(NCT02340975) (MEDI4736) [1, 2]	CTLA-4	Tremelimumab	2	GC 2nd line	18	A partial response was observed in one patient while steady progression was observed in four patients. The adverse effect such as CEA.TR-TEAEs: ≥ 3 TR-TEAE, Fatigue, thrombocytopenia, and anemia were also observed. Autoimmune hepatitis and hepatic failure were observed in one treatment-related death patient.
NCT02267343[3]	CTLA-4	Ipilimumab	3	Advanced Gastric or Gastroesophageal Junction Cancer cohort	330	8.87 months (IQR 6.57-12.37) was the follow-up median for surviving patients in the nivolumab group, 5.26 months with (95% CI 4.60-6.37) is the overall survival median in the nivolumab group. The overall survival rate of 26.2% was observed within the nivolumab group compare to 10.9% within the placebo group. There are grade 4 treatment-related adverse effect in 10% of the 330 patients from the nivolumab
(NCT01848834) (NCT02370498) (NCT01848834) (NCT02335411) [4-11]	PD-1	Pembrolizumab	1b	PDL1+ GC Cohort	39	Central review: ORR 22% median response period of 40-weeks. Overall response rate: 11.4 months. Progression-free survival: 1.9 months.PD-L1 association and overall response rate by central and investigator review was 22% (95% CI 10-39) and 33% (95% CI 19-50) respectively. Eight weeks (range 7-16) was recorded as the median period of response. An overall survival of 69% over the period of 6-month was recorded with a PD-L1 expression level progressive survival rate (PFS) of 24% over a period of 6-month was associated with ORR (1-sided P = 0.10).
(NCT01772004) (MSB0010718C) [2,12]	PDL-1	Avelumab	1b	Japanese GC All comers 2 cohorts	75 (20-second line and 55-maintenance)	Central review: ORR. 15 % and 7.3 % progression-free survival over the period of 11.6 weeks and 14.1 weeks respectively. Progression-free survival was observed in 7 patients out of the 20-second line patients, and 43 out of the 55 maintenance patients with evaluation of PD-L1 expression level. In the second line group of 20 patients, a Progression-free survival median of 36.0 wks (95% CI: 6.0, 36.0) was observed for PD-L1+ expression level and 11.6 wks (2.1, 21.9) for PD-L1 expression level. Furthermore, in the maintenance group of 55 patients, a Progression-free survival median of 17.6 wks (5.9, 18.0) was observed for PD-L1+ expression level and 11.6 wks (5.7, 17.7), for PD-L1 expression level.
(NCT01693562) (MEDI4736) [13, 14]	PDL-1	Durvalumab	1	Gastric Cancer cohort with none specified PD-L1 positive status	16 of 346	Central review: ORR of 25 % PD-L1 association and response.
MPDL3280A (MPDL3280A) [15-17]	PDL-1	Atezolizumab	1	GC cohort All comers	1 of 171	Partial response in one patient was observed with a PD-L1 association and PFS of 42% within 24 weeks. PD-L1 expression level status was evaluated in 94 patients who had a tumor. The PD-L2 EXPRESSION LEVEL evaluation was done in 81 patients with an expression twice higher than PD-L1 expression from positive tumor patients compared to the negative PD-L1 tumor patients. Thirteen out of the 33 patients had an ORR of 39% with positive PD-L1 expression level compare to the 13% (8/61) of patients who expressed negative PD-L1 in neg tumors.

ORR; overall response rate, MOS; median overall survival, PFS; median progression-free survival, w; week, m; month

Table 2. Characteristic of ongoing immune checkpoint clinical trials in gastric cancer.

Study/ Clinical trial identification/Refe	Clinical trial Phase	Strategy	Status	Indication
Roy S et al., 2016/published/(69)	1	Pembrolizumab in combination with ramucirumab	Recruiting	Defined group, second or third line regimen
Philip et al., 2016/published/(70) NCT02563548	1	Pembrolizumab in combination with PEGPH20	Recruiting	Defined group, at least second line
Muro et al., 2016/published/ NCT01848834 (KEYNOTE 012)(71)	1	Pembrolizumab	Active, not recruiting	Defined group ,non- relapsed setting
Zev et al., 2016/published/ NCT02452424(72)	1	Pembrolizumab in combination with PLX3397	Recruiting	Defined group , non- relapsed setting
Karen et al., 2016/published/ NCT01772004/(73)	1	Avelumab	Recruiting	Defined group, third line
Yasuhide et al., 2016/published/ NCT01943461/(74)	1	Avelumab	Recruiting	second and third line, Asian and Japanese
Howard et al., 2017/published/ NCT01633970/(75)	1	MPDL3280A with bevacizumab administered as monotherapy or as combined therapy	Recruiting	Basket trial
David et al., 2016/published/ NCT01375842/(76)	1	MPDL3280A	Recruiting	Basket trial
Jessica et al., 2016/published/ NCT02471846/(77)	1	MPDL3280A and GDC-0919	Recruiting	Defined group , non- relapsed setting
2016/published/ NCT02318901	1/2	Pembrolizumab with trastuzumab as combination	Recruiting	Defined group, human epidermal growth factor receptor 2) - positive
2016/published/ NCT02268825	01/2	Pembrolizumab in combination with FOLFOX	Recruiting	Defined group
Ronan et al., 2016/published/ NCT02340975(78)	1b/2	Tremelimumab MEDI4736	Recruiting	non- relapsed setting
Jedd et al., 2016/published/ NCT01585987(79)	2	Ipilimumab versus. FU/BSC	Recruiting	Maintenance after 1st line
Antonia et al., 2016/published/ NCT01928394(80)	1/2	Nivolumab +/- ipilimumab	Completed	Defined group , non- relapsed setting
Jeed et al., 2016/published/ NCT02488759(81)	1/2	Nivolumab	Recruiting	Epstein–Barr virus -positive
Josep et al., 2016/published/ NCT02494583 (KEYNOTE 062)(82)	3	Pembrolizumab monotherapy or combination with CT	Recruiting	1st Line, HER2-negative, PDL1-positive
Atsushi et al., 2016/published/ NCT02370498 (KEYNOTE 061)(83)	3	Pembrolizumab verse. paclitaxel	Second line	Enrolling

Clinical trial studies of PD-1 blockade approach in gastric cancer

It is evident within cancers evolutionary pressure exists to avoid attack by the immune system. To this end cancer cells can employ signals the body uses to control self-damaging autoimmunity. Especially PD-1 appears prominent in limiting immunity towards cancers cells. In physiology, PD1 guards against autoimmunity through a dual mechanism of promoting programmed cell death in cytotoxic T-cells while simultaneously reducing apoptosis in regulatory T cells. By engaging PD1 on anti-tumor immune cells through its cognate ligands, in particular, PD-L1 the cancer cell can corrupt anti-oncogenic immunity. Anti PD1 antibodies prevent this interaction by binding to the relevant immune cells. Accordingly, anti-PD-1 antibodies have shown such anticancer efficacy in various cancers types[6]. Concerning this strategy, its safety and effectiveness were tested with pembrolizumab is patients from the Asian-Pacific patients who presented with progressive gastric or gastroesophageal junction tumors and exhibited a positive PD-L1 expression status. Concerning the latter, the eligibility criteria were that patients should have or $\geq$ 1% cancer nest cell PD-L1 staining. The design involved a gift of 10 mg of pembrolizumab per kg once in two weeks for one year or until comprehensive response, advancement or unacceptable toxicity. The primary efficacy endpoint was evaluated after eight weeks medical imaging, whereas duration of response constituted a secondary endpoint. In this study, the overall response was 22% (95% CI, 10.39) and 33% (95% CI, 19.50) respectively, which although an encouraging result not markedly better as compared to studies employing unselected patients. Hence, the PD-L1 expression per se is suitable for selecting gastric cancer patients for PD-1-directed checkpoint therapy remains poor and other approaches need to be explored.

In a clinical trial NCT02267343[19], involving the use of Nivolumab on gastro-oesophageal junction or advanced gastric cancer refractory patient, the cohort with intolerant of, at least two preceding treatment regimens were recruited in a placebo-controlled, double-blinded randomized clinical trial involving 493 patients who were assigned randomly to take nivolumab. 8.87 months (IQR 6.57-12.37) was the follow-up median for surviving patients in the nivolumab group. 5.26 months with (95% CI 4.60-6.37) is the overall survival median in the nivolumab group. The overall survival rate of 26.2% was observed within the nivolumab group compare to 10.9% within the placebo group. There is grade 4 treatment-related adverse effect in 10% of the 330 patients from the Nivolumab arm of the study[19].

Apart from targeting PD-1 on immune cells, inhibiting its cognate ligand PD-L1 on the tumor proper constitutes a therapeutic possibility. Various studies have addressed this possibility by testing the efficacy and performance of Avelumab (NCT01772004 (JAVELIN), MSB0010718C) in chemotherapy-failing gastric cancer patients[6]. Twice every month, patients were given 10 mg/kg of avelumab until either progression, or intolerable toxicity occurred. Cancerous growth, OR, adverse events and PFS were evaluated once every six weeks, and results were related to PD-L1 expression as determined by immunohistochemistry. In total, 75 Gastric cancer patients were treated with avelumab. Of these patients, seven showed a response and of these seven three were positive for PD-L1 as determined by histochemistry. It thus appears that PD-1 is the more attractive target for therapy in gastric cancer as compared to PD-L1 and again attempts at precision medicine, employing PD-L1 immunoreactivity as triaging agent mostly fail. In this context, it is interesting to note that in such studies PD-L1 expression is usually determined on material collected before the onset of conventional treatment. One can well imagine that chemotherapy etc. influences the expression of checkpoints. We thus call for studies investigating the potential of PD-L1 immunohistochemistry for selecting potential responders on material obtained immediately before anti-checkpoint therapy is initiated.

Clinical trial studies of CTLA-4 blockade approach in gastric cancer.

Apart from targeting the PD1/PD-L1 signaling system, CTLA4 is also a prominent immuno-checkpoint. CTLA4 is an immune checkpoint receptor that downregulates immune responses. The molecule is constitutively expressed in regulatory T cells but only upregulated in conventional T cells after activation. Ipilimumab targeted CTLA4 and was the first checkpoint inhibitor to reach the clinic, meeting substantial clinical success in metastatic melanoma. Results in gastric cancer were somewhat less encouraging. A recent study employing ipilumimab in unresectable Locally Advanced/Metastatic Gastric or Gastroesophageal Junction Cancer did not provide evidence ipilimumab efficacy as monotherapy[20]. Another CTLA4-targeting antibody is tremelimumab. In this study, a partial response in one patient, and stable disease in four others (out of 18 participants) was seen, which does not compare well with results obtained with PD-1-directed strategies. Hence, the usefulness of CTLA4 immuno-checkpoint targeting is doubtful, although currently also combinations between anti-CTLA4 and anti-PD1 are being tested, which may still uncover potential synergism.

Adverse events

It is pertinent to note that immune-modulating agents can exert some levels of toxicity[21, 22] and anti-immuno-checkpoint therapy of gastric cancer is not an exception. Unfavorable event outcomes were seen in several clinical trials which can be managed by the administration of corticosteroids although it is an immunosuppressive agent. Colitis resulting from checkpoint inhibitor therapy is sometimes managed by infliximab, an anti-tumor necrosis factor alpha agent. At the latter compound provokes T cell apoptosis, this is probably detrimental and should be avoided[23]. In a clinical testing involving pembrolizumab and Avelumab, a five total grade drug-related adverse event was observed in four patients, including loss-of-appetite, fatigue, damage to peripheral nerves and peripheral ischemia. However, no fatalities as consequence of drug side-effects have been reported, and it is fair to say that although not little off-target effects can be efficiently clinically managed.

Conclusions

PD-1 inhibition is a useful clinical option for advanced gastric cancer patients failing other modes of treatment and performs better other forms of checkpoint inhibitor therapy in this disease and associated with acceptable toxicity. At present strategies to target anti-PD1 therapy to those patients most likely to benefit from such intervention based on tumor PD-L1 expression have largely failed. Hence the reason why only a minority of gastric cancer patients benefit from anti-PD1 therapy remain primarily obscure and require further investigation

Competing interests

No competing interests.

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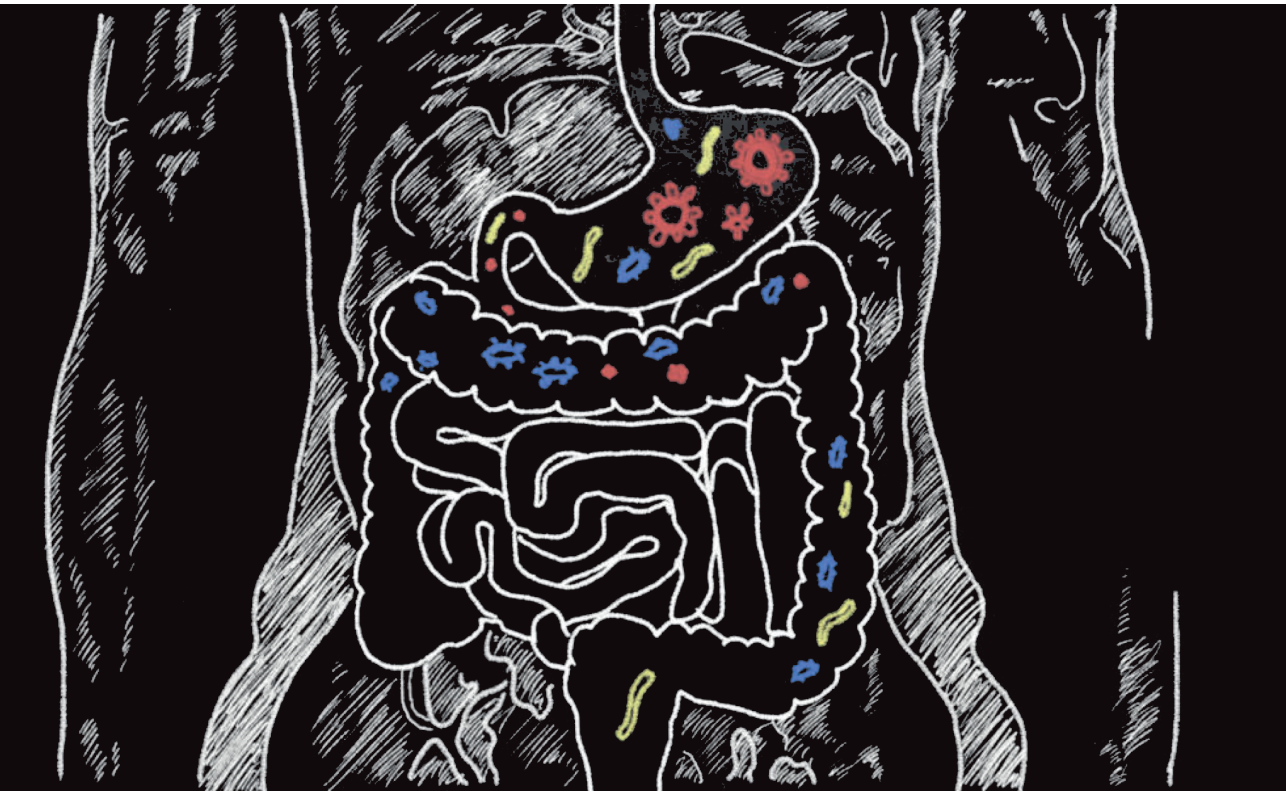

Chapter 4

Smo-dependent and independent pathways in non-canonical Hedgehog signaling

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Abstract

Hedgehogs constitute a family of pivotal morphogens that exert their action through a well-characterized canonical pathway involving Gli transcription factors and as yet poorly characterized non-canonical signaling. To obtain more insight in the non-canonical aspect of Hedgehog signaling, we perform a comprehensive characterization of the cellular kinome in Hedgehog-challenged wild-type, *Ptc*$^{-/-}$, Smo$^{-/-}$, and Smo-agonist Purmophamine-challenged wild-type fibroblasts. We observe that whereas non-canonical Hedgehog signaling in general displays an absolute requirement for the presence of functional *Ptc*, an elaborate signaling network of both *Smo*-dependent and independent pathways is present that mediates actin reorganization through the activation of Src-like kinases, activates various proinflammatory signaling cascades and concomitantly canonical Wnt and Notch signaling while suppressing canonical BMP signaling. The observation that Ptc-dependent Smo-independent non-canonical Hedgehog signaling increases Wnt/Notch provides an obvious explanation for the failure of Smo antagonists in combating gastrointestinal cancer and suggests that therapy should be directed at inhibiting Hedgehog-Ptc interaction.

Keywords: Sonic; Hedgehog; Transcription; Cytoskeleton; Endocytosis.

Introduction

Cell fate in the developing or adult body is determined by morphogens, molecules whose non-uniform distribution governs the pattern of tissue development often by providing positional information[1, 2]. Notable examples of morphogens include the Hedgehog-, Wingless-related integration site- (Wnt) and Bone morphogenetic protein- (BMP) dependent signals[3-6]. The intracellular signaling resulting from engagement of morphogens with their cognate receptors is involved in virtually all physiological and pathophysiological processes in the body, including embryogenesis, tissue regeneration, and carcinogenesis and thus understanding morphogen signaling is a scientific question of utmost importance[7, 8]. Unfortunately, morphogen-provoked signal transduction is often complex, and a special case to the point is signal transduction initiated by Hedgehogs[9].

Hedgehog proteins are a highly conserved family of intercellular signalling molecules. Originally identified as a Drosophila segment polarity gene required for embryonic patterning, several vertebrate homologues have now been discovered—Indian (Ihh), Desert (Dhh) and Sonic Hedgehog (Shh), the most extensively characterised[10]. Hedgehog signals are fundamental regulators of embryological development, as illustrated by the dramatic embryological malformations seen when accurate timing of Hedgehog signals during gestation is corrupted[11]. Hedgehog remains active in the post-embryonic period, maintaining histostasis in a variety of tissues, including the gastrointestinal tract and the immune system[12]. Continuous hedgehog signalling is an essential permissive factor for many cancers and causative in basal cell carcinoma of the skin amongst others[13]. In humans, one-allelic loss of the inhibitory hedgehog receptor Patched is sufficient to produce the so-called Gorlin syndrome [14], associated with multiple basal cell carcinomas. Despite the importance of hedgehog signalling for human physiology and pathophysiology, the molecular details underlying this signalling remain only partly characterised.

Hedgehog signal transduction is highly unusual, containing many features unique to this signaling system[15]. After synthesis, Hedgehog undergoes autocatalytic cleavage followed by lipid modifications resulting in the only protein in the animal kingdom that is sterolated and both secreted and palmitoylated[16]. The primary receptor for Hedgehogs is Patched, an intriguing receptor, as it does not convey the Hedgehog signal to the intracellular components of the pathway itself like a conventional receptor. Rather, binding of Hedgehog to Patched alleviates the

inhibitory effect of Patched on another membrane receptor, Smoothened. The alleviation of Patched inhibition is probably caused by internalization of Patched following binding of Hedgehog, but the signaling mechanisms involved remain obscure at best[2, 17]. Following removal of Patched-mediated repression Smoothened translocates to the primary cilium in a microtubule and kinesin-dependent process, that is essential for Smoothened to initiate further signaling as evident from the potential from pharmacological inhibitors of this process to counteract canonical Hedgehog signaling[18, 19]. Subsequently, Smoothened mediates the activation of the latent transcription factor glioma-associated oncogene (Gli) via a process which is still largely unresolved but involves the kinase Fused (Fu) and the Suppressor of Fused protein (Su(Fu))[20, 21] . Gli proteins are the final transcriptional effectors of Hedgehog signaling, both in normal vertebrate development as well as oncological disease[22, 23]. Together this signalling cascade may be termed the canonical hedgehog pathway. It is obvious that enhanced knowledge of the signaling elements involved in this pathway should prove exceeding useful in defining novel rational therapy directed at disease emanating from aberrant activation of canonical Hedgehog signaling.

Apart from canonical Hedgehog signaling, Hedgehog effects in physiology and pathophysiology also depend on so-called non-canonical signaling. For most morphogens, non-canonical signaling has been identified and effects observed are in general contrasting the effects derived from canonical signaling. An example is BMP signaling. In the colon BMP generally acts as a tumor suppressor[24]. In presence of canonical BMP-signaling abrogating SMAD4 mutations, however, a non-canonical BMP-induced signaling pathway becomes evident that stimulates epithelial-to-mesenchymal transition and metastasis via activation of Rho and ROCK and furthers the colon cancer process[25]. Likewise, non-canonical Wnt signal transduction mediates important aspects of the action of this morphogen in the body through activation of small GTPases like Rac, Rho and Cdc42 to regulate the activity of ROCK, MAPK and JNK as well as Ca++ signaling, also an effect important for colon cancer metastasis[26-28] . For Hedgehog also various modes of non-canonical signaling have been described, both downstream of Patched and independent of Smoothend as well as downstream of Smoothened. The most prominent example of the former concerns colorectal cancer stem cells[29]. Whereas canonical Gli-dependent Hedgehog signaling negatively regulates Wnt signaling in the normal intestine in and intestinal tumors[7, 30], Hedgehog signaling in colon cancer stem cells activates a non-canonical Patched-dependent but

Smoothened independent signaling that is required for survival of these cancer stem cells[29]. Tantalizingly, these results open the theoretical possibility to uncouple the anti-cancer effect of Hedgehog signaling on colorectal cancer in general[31] and the trophic effect of Hedgehog signaling on specifically colorectal cancer cells. In the absence, however, of knowledge on the molecular pathways that mediate these non-canonical effects of Patched-dependent but Smoothened independent Hedgehog signaling, this possibility remains hypothetical only.

Apart from Patched-dependent Smoothened-independent non-canonical Hedgehog signaling, Smoothened-dependent Gli-independent non-canonical Hedgehog signaling has also been described and likewise the molecular mechanisms involved are only partly understood. The interaction of Hedgehog with Patched stimulates the translocation of Smoothened to the primary cilium, which is required for the transcriptional Hh response. This translocation involves activation of phospholipase A2 following Smoothened activation and results in the enzymatic release of arachidonic acid from plasma membrane phospholipids. The thus released arachidonic acid binds to Smoothened and this interaction appears essential for cilium translocation of Smoothened and canonical Hedgehog signaling[32]. Arachidonic acid metabolites are powerful actin cytoskeleton remodeling agents[33] and while located outside the primary cilium Smoothened also mediates transcription-independent actin reorganization and chemotactic responses through the production of these metabolites[16, 34]. The physiological importance of this non-canonical response to Hedgehog signaling is illustrated by its pivotal role in Hedgehog effects in directing neurite projection[35], It has become clear, however, that non-canonical Hedgehog effects on axonal guidance involve activation of Src-like kinases[36] but generally speaking it is fair to say that the molecular events underlying Smoothened-dependent non-canonical signaling remain unresolved.

The above-mentioned considerations prompted us to characterize the kinase activities associated with Hedgehog challenge in general as well as those specifically associated with Patched activation and Smoothened activation in isolation. To this end we exploited the power of the peptide array-based kinome profiling in which peptide arrays of kinase-specific substrates are incubated with cell lysates and 33P-γ-ATP thus generating comprehensive descriptions of cellular kinase activities[37, 38]. The results provide a wealth of information on the effects of Hedgehog signal transduction on cellular phosphorylation events and reveal a Patched-dependent Smoothened-independent signal transduction cascade that

amongst other events provokes Wnt and Notch activation, the former probably through stimulation of phosphoinositide-3-OH-kinase (PI3K), stimulates the activation of various pro-inflammatory signaling events and results in the activation of protein kinase A (PKA), a potential feed forward inhibitory event with respect to canonical Hedgehog transduction. Smoothened-dependent events include inhibition of PKA, stimulation of various cytoskeleton reorganizing pathways as well as stimulation of AMP-activated kinase (AMPK). In conjunction, the results provide an intellectual framework that allows understanding of context-dependent Hedgehog signaling.

Experimental procedures

Materials

Cyclopamine was from Biomol. Purmorphamine was from EMD Biochemicals (Darmstadt, Germany). Recombinant ShhN was from R&D Systems

Cell culture

Smoothened$^{-/-}$ fibroblasts (a gift from Dr. Taipale), Ptch1$^{-/-}$ and wild-type MEFs (gifts from Dr. Scott) were cultured in Dulbecco's modified Eagle's medium (DMEM, Invitrogen) supplemented with 10% fetal calf serum (FCS, Invitrogen). For experiments, a confluence of 50% cells was allowed to grow in six-wells plates. Stimulation were done, if appropriate with two µg/ml Shh for 10 min.

Kinome Profiling

For peptide array analysis, we employed the Pepchip kinomics array. The protocol and associated analysis has been described in detail elsewhere[55] and is based on the original protocol of van Baal et al[56]. In short, cells were washed in ice-cold PBS and lysed in a non-denaturing complete lysis buffer (cells were lysed in 50µl lysis buffer (20mM Tris-HCl pH 7.5, 150mM NaCl, 1mM EDTA, 1mM EGTA, 1% Triton X-100, 2.5mM sodium pyrophosphate, 1mM MgCl2, 1mM glycerophosphate, 1mM Na3VO4, 1mM NaF,1µg/ml Leupeptin, 1µg/ml Aprotinin, 1mM PMSF). Subsequently the cell lysates were cleared by centrifugation and peptide array incubation mix was produced by adding 10 µl of activation mix (50% glycerol, 50 µM ATP, 0.05% v/v Brij-35, 0.25 mg/ml bovine serum albumin) and 2

µl [γ-33P] ATP (approx. 1000 kBq (Amersham AH9968). Next, the peptide array mix was added onto the chip, and the chip was kept at 37°C in a humidified stove for 90 min. Subsequently the peptide array was washed twice with Tris-buffered saline with Tween 20, twice in 2 M NaCl, and twice in demineralised H2O and then air-dried. The chips were exposed to a phosphor screen for 72 hours, and the density of the spots was measured and analyzed with array software (ScanAnalyze). ScanAlyze software was used. Using grid tools, spot density and individual background were corrected and spot intensities and background intensities were analyzed. Data from at least 9 independent data points were exported to an excel sheet for further analysis. Control spots on the array were analyzed for validation of spot intensities between the different samples. Inconsistent data (i.e., SD between the different data points >1.96 of the mean value) were excluded from further analysis. For each peptide the average and standard deviation of phosphorylation was determined and plotted in an amplitude-based hierarchical fashion. For data analysis, first every peptide was given an "on" call or "off" call (Markov state analysis). To this end, first an average signal was calculated for each peptide using the three biological replicates (each consisting of two technical replicates) yielding an aggregate dataset for each the hematopoietic subsets. Subsequently, for each of the aggregate datasets, either "on" calls or "off" calls were given to each peptide substrate (Markov state analysis). In order to do this, we assumed that the subset of signals representing the 1-1-e fraction of peptides having the lowest phosphorylation of all peptides contained pure noise and did represent meaningful phosphorylation. The distribution of this noise was fitted as a single exponent, using the amplitude-sorted row number of these substrates as the X domain of the distribution and this single exponent was assumed to describe noise for the entire dataset. Now for all data points within the subset, when the actual amplitude observed minus 1,96 the standard deviation was in excess of the value expected from distribution describing the noise, a substrate was given an "on" cal ($p < 0.05$) in this Markov analysis. Subsequently results were collapsed on elective signal transduction categories and subjected to dichotomal significance analysis, contrasting Shh-stimulated cultures to parallel vehicle cultures or Purmorphamine-stimualted cultures to parallel unstimulated cultures. If a significant result ($p < 0.05$) was detected, we considered the result as robust evidence of differential activation of signal transduction between Hedgehog-stimulated and unstimulated cultures and in the depiction of results the corresponding signal transduction categories have been highlighted with a red border. For those signal transduction categories in which

using this dichotomal testing based on number of Markov state "on" peptides did not result in statistical significance, the relative levels of phosphorylation were also tested using a paired T test, directly parametrically comparing phosphorylation of the corresponding spots. As we considered thus-discovered statistically significant differences between the relevant experimental conditions less robust, in the depiction of the results they have been highlighted with an orange border. Note that due to differences in the number of peptides allotted to the signal transduction categories apparently large differences in phosphorylation not always yield statistically significant results, while smaller differences can produce such results if the number of substrates in such categories is large.

Results and discussion

Hedgehog stimulation provoked rapid and marked reorganization of the cellular kinome

The general approach as to this study, both technically and biologically is provide through Figure 1. We characterised the kinase signatures associated with Hedgehog stimulation of mouse embryonic fibroblasts (MEFs), which we have recently shown to constitute a powerful model for delineating signal transduction events[39]. We established that under our experimental conditions, these cells do not endogenously release Hedgehog (not shown). Cells were incubated for 10 min with either 1 µg/ml Shh or a vehicle control, and the cell lysates were employed for in vitro phosphorylation of peptide arrays using 33P-γ-ATP. Arrays consisted of 1024 different undecapeptides, of which 48 are various technical controls, whereas the remaining 976 peptides provide kinase substrate consensus sequences spanning the entire mammalian kinome and which we have shown earlier to provide comprehensive insight in cellular signal transduction[40]. On each separate carrier, the array was spotted three times, to allow assessment of possible variability in substrate phosphorylation. The final physical dimensions of the array were 25 x 75 mm, each peptide spot having a diameter of approximately 250 µm, and peptide spots being 620 µm apart. As a control for the specificity of the reaction 33P-□-ATP was used; no incorporation of radioactivity was seen. We then calculated the mean phosphorylation level for all substrates before and after the treatment (total number of data points is 9 for each group). The technical quality of the profiles was good, and we only allowed experiments in which the Pearson product moment correlation coefficient was more as 0.95 for the technical replicas. We decided to use these data

for analysis as to specific and divergent phosphorylation following stimulation with Hedgehog. To this end results were collapsed on elective signal transduction categories (see experimental procedures and [41]. The results are shown in Figure 2 and detailed in Supplementary table 1. They show that Hedgehog challenge provokes fast and substantial remodeling of cellular signaling. Particularly notable is the upregulation of mTOR signaling which may fit recent data that indicate that mTORC1-mediated translation is a key component of hedgehog signaling and is a putative target for treating Hedgehog-driven cancers[42]. Other interesting points include an upregulation of g-protein-coupled receptor kinase enzymatic activity, which is line with observation in Drosophila that a G-protein-coupled receptor kinase controls Smoothened activity[43]. Strong regulation of PKA is also observed and may be in line with the notion that this kinase is a regulator of Hedgehog signaling[44], whereas the observation that PKC enzymatic activity is upregulated is conform the canonical mode of action of G-protein coupled receptor like Smoothened. A variety of pro-inflammatory signaling modules is activated as well (including Lyn, Fyn and peptides that are consensus substrates for Bruton's tyrosine kinase) but as embryonic fibroblasts are not immunologically-relevant cells, the importance of this observation is uncertain. Maybe unsurprisingly (in view of its role of a Wnt antagonist in the epithelium of the intestine[7, 30], we see strong inhibition of Wnt signaling in our model system following Hedgehog application although this observation is at bay with the data of Shin et al. that show that bladder stromal cells respond to Shh with Wnt activation[45] and also to the recent report of Regan et al.[29] describing that Hedgehog can stimulate Wnt signaling in cancer stem cells. In addition, the upregulation of substrate peptides for p21-activated kinase (Pak) activity and related molecules indicates that Hedgehog stimulation stimulates actin reorganization and morphological changes. In conjunction these experiments show that the effect of Hedgehog on the cellular kinome is rapid and profound.

Patched-dependent Smoothened-independent effects on cellular kinase activity

The existence of Patched-dependent Smoothened-independent signal transduction is supported by various observations[46] and appears highly relevant in that it is essential for cancer stem cell survival in colorectal cancer[29]. To test whether such signaling is present in our model system we incubated MEFs with 3H-sucrose (which is membrane impermeable and is only taken up via endocytosis in most cell types) and challenged the cells with either a vehicle control or 1 µg/ml Shh, in the

presence or absence of the Smoothened inhibitor cyclopamine (Figure 3). We observed strong accumulation of radioactivity in both Hedgehog challenged cells, as well as in cells challenged with Hedgehog in the presence of cyclopamine. We thus concluded that endocytosis following Hedgehog stimulation does not require Smoothened activity and that hence our model system was suitable for investigating at least certain aspects of Smoothened-independent signal transduction. To further characterize these aspects we performed kinome profiling of Smoothened-/- fibroblasts (originally obtained from Drs. James Chen and Philip Beachy and previously described by Varajosalo et al[47], challenged with either a vehicle control or 1 µg/ml Shh for 10 min. The results are summarized in Figure 3 and Supplementary table 1 and reveal that the influence of Smoothened-independent Hedgehog-induced signaling on cellular kinase activity is substantial. Conspicuously lacking, however, is G protein-coupled receptor-associated signal transduction which is obviously in line with the absence of Smoothened-dependent events. In particular activation of cytoskeletal remodeling is seen following addition of Hedgehog, which correlates with a downregulation of activity of the Csk negative regulators of Src activity and may relate to the observed Smoothened-independent effects of Hedgehog on endocytosis described above, especially as kinase enzymatic activity directed against FAK-responsive peptides is co-activated[48, 49]. A prominent effect is increased mTOR activation whereas inflammatory signal transduction was also activated by Hedgehog in Smoothened-/- fibroblasts. Hedgehog in wild type fibroblasts provokes similar effects (see above) and thus these effects of Hedgehog signaling appear thus at least partially to stem from Smoothened-independent signaling. Similarly, activation of Wnt and Notch signaling is also seen and thus this aspect of Hedgehog signaling seems also independent of Smoothened. Interestingly, in the absence of Smoothened, Hedgehog activates rather than inhibits PKA, and it is tempting to speculate that this effect may relate to activating phosphorylation of Smoothened by PKA that has been described in Hedgehog signaling[50]. Control experiments revealed that the effect of Hedgehog on Patched negative fibroblasts was negligible (although generally speaking Patched-negative fibroblasts show more kinase activity as compared to control cells, potentially an effect of unleashed Smoothened signaling), suggesting that a contribution of Patched-independent Hedgehog-dependent signaling is non-existent (Supplementary table 1). In conjunction these results reveal that an unexpectedly large proportion of Hedgehog signal transduction towards the cellular kinome is mediated though non-canonical Patched-depedent Smoothened-independent signaling.

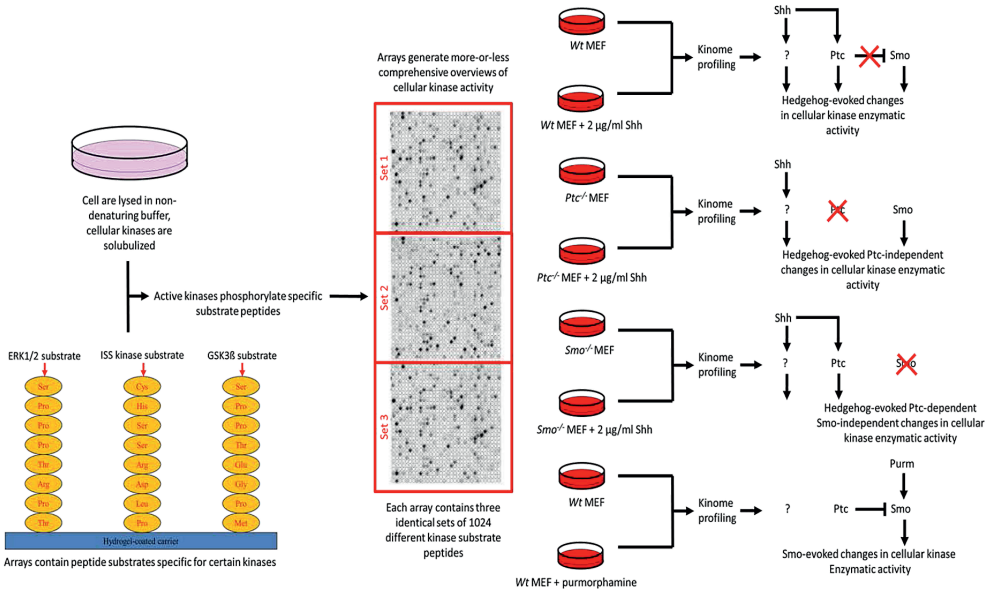

FIGURE 1. Outline of the study. A. Technical approach – kinome profiling. In this study, we aim to comprehensive characterize cellular kinase enzymatic activities. To this end appropriately stimulated cell cultures are washed with ice-cold PBS and lysed in a non-denaturing complete lysis buffer as to solubilize cellular kinases. Lysates are the transferred to arrays consisting of a substrate peptide library, spotted in triplicate as to assess technical reproducibility, which are spotted on a hydrogel-coated glass carrier. Upon addition of radioactive ATP and an activation mix, kinases –if enzymatically active- will phosphorylate substrate peptides. Incorporation of radioactive ATP into a substrate peptide is taken as a measure of enzymatic kinase activity towards a particular substrate. The broad variation in specific substrates used (see also supplementary data) allows obtaining a more-or-less complete description of cellular signaling, the so-called kinome. B. Biological approach. In this study, we first generate a description of the effects of Shh challenge on cellular signaling in general by comparing kinome profiling results of cultures challenged and not challenged by the morphogen. To delineate the contribution of possible non-Ptc-dependent components to Hedgehog-dependent kinase events, the effect of Shh stimulation on fibroblast genetically devoid of Ptc is established as well. To identify signal transduction events that are downstream of Ptc but do not involve Smo, the Hedgehog provoked effects on the cellular kinome are studied in fibroblasts genetically deficient for Smo. Finally, to identify events that are solely dependent on the activation of Smo, we study the effects of the Smo agonist purmorphamine

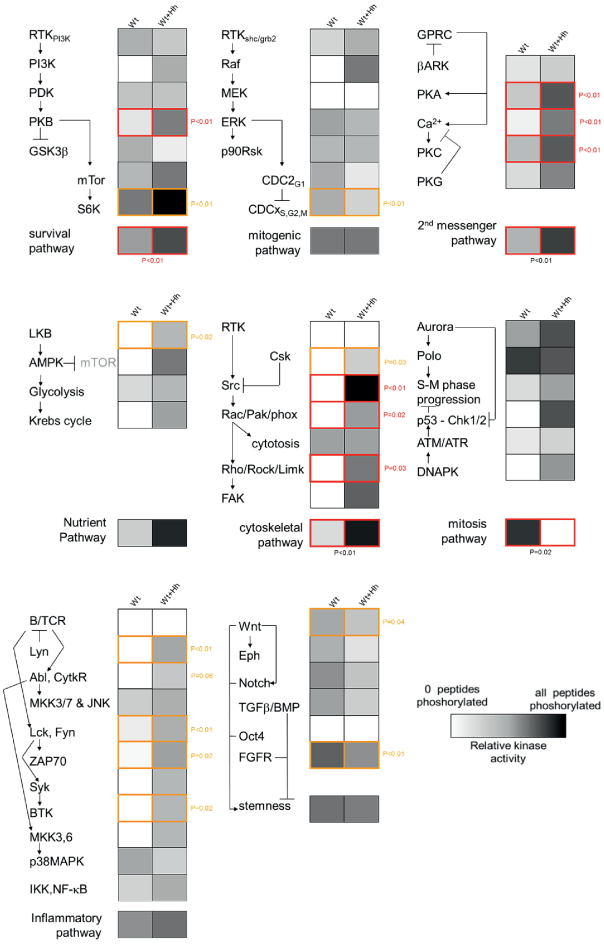

FIGURE 2. Effects of Hedgehog stimulation on cellular signaling as determined by kinome profiling. Murine fibroblasts were stimulated with 1 µg/ml Shh. Subsequently cells were lysed and the resulting lysates were used to phosphorylate arrays of different kinase substrates employing 33P-□ATP and radioactivity incorporated in the different substrates was determined. Peptide substrates were allotted to elective signal transduction elements. The picture depicts the number of peptides significantly phosphorylated (which means the number of peptides that received a Markov "on" call - see experimental procedures) for each element. A darker color reflects more kinase activity towards substrate elements and the results reveal the effects of Hedgehog stimulation on cellular signal transduction, thus a black color means all peptides were significantly phosphorylated, whereas a white color means that no peptides allotted to this signal transduction in this experimental conditions were phosphorylated. Results were first statistically tested by a dichotomal analysis based on the number of Markov "on" calls observed in vehicle-and Shh-stimulated cultures. If statistically significant differences were noted the signal transduction category is highlighted with a red border and the level of significance observed is indicated in red. For signal transduction elements in which this very robust analysis fails to detect a statistically significant difference, a parametric test was performed. If this proved significant, the category is highlighted with an orange color and corresponding level of significance is depicted as well – in orange. The results provide a wealth of data on the effects of Hedgehog stimulation on cellular signaling.

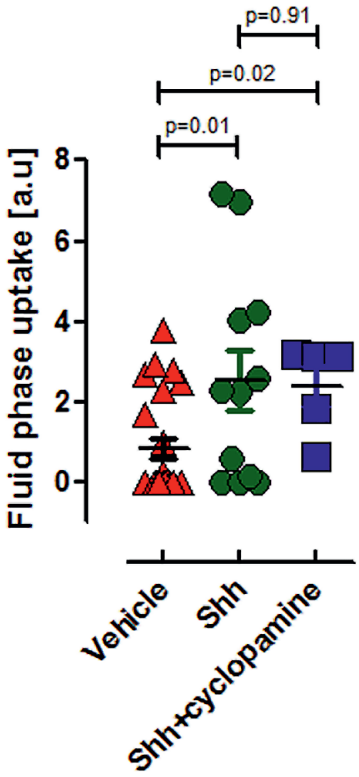

FIGURE 3. Effects of Hedgehog on the uptake of [3H]-sucrose and the influence of Smoothened inhibition thereon. Fibroblast cultures were grown in twenty-four-wells plates and incubated in a 1 ml containing 200 nCi of [3H]-sucrose in the presence or absence of either 1 µg/ml Shh and 10 µM cyclopamine. At the end of the experiment cells were extensively washed with ice-cold PBS and lysed in NP-40 for subsequent scintillation counting, As sucrose can only enter cells through fluid phase uptake, this provides a reliable measure of cellular endocytosis. We observe that Hedgehog stimulates fluid phase uptake and this effect does not require Smoothened as it is not sensitive to the Smoothened inhibitor cyclopamine

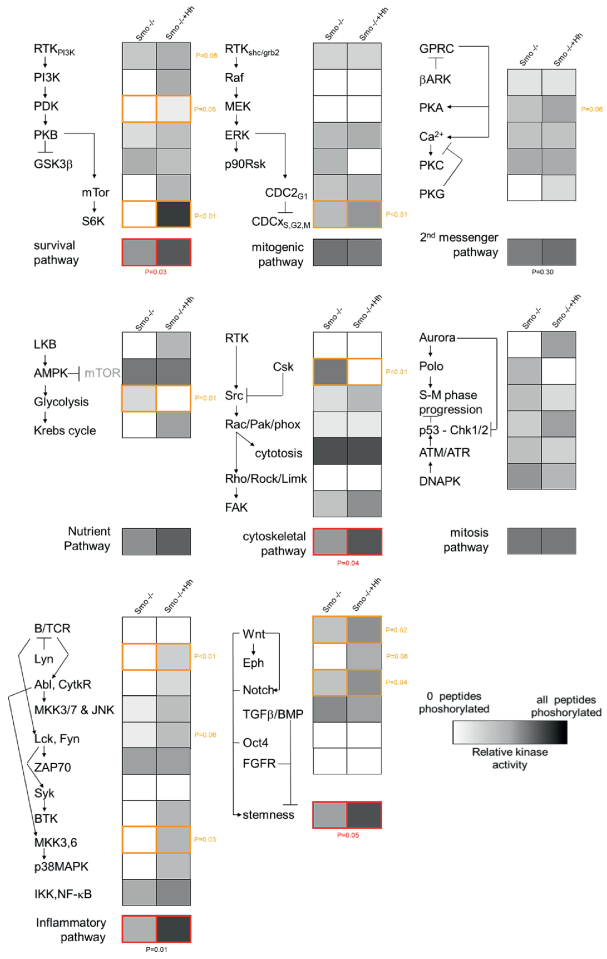

FIGURE 4. Effects of Hedgehog stimulation on cellular signaling in Smo-deficient fibroblasts. Murine Smo-/- fibroblasts were stimulated with 1 µg/ml Shh. Subsequently cells were lysed and the resulting lysates were used to phosphorylate arrays of different kinase substrates employing 33P-□ATP and radioactivity incorporated in the different substrates was determined. Peptide substrates were allotted to elective signal transduction elements. The picture depicts the number of peptides significantly phosphorylated (which means the number of peptides that received a Markov "on" call - see experimental procedures) for each element. A darker color reflects more kinase activity towards substrate elements and the results reveal the effects of Hedgehog stimulation on cellular signal transduction, thus a black color means all peptides were significantly phosphorylated, whereas a white color means that no peptides allotted to this signal transduction in this experimental condition were phosphorylated. Results were first statistically tested by a dichotomal analysis based on the number of Markov "on" calls observed in vehicle-and Shh-stimulated cultures. If statistically significant differences were noted the signal transduction category is highlighted with a red border and the level of significance observed is indicated in red. For signal transduction elements in which this very robust analysis fails to detect a statistically significant difference, a parametric test was performed. If this proved significant, the category is highlighted with an orange color and corresponding level of significance is depicted as well – in orange. The results reveal an intricate web of Patched-dependent Smoothened-independent non-canonical signal transduction events.

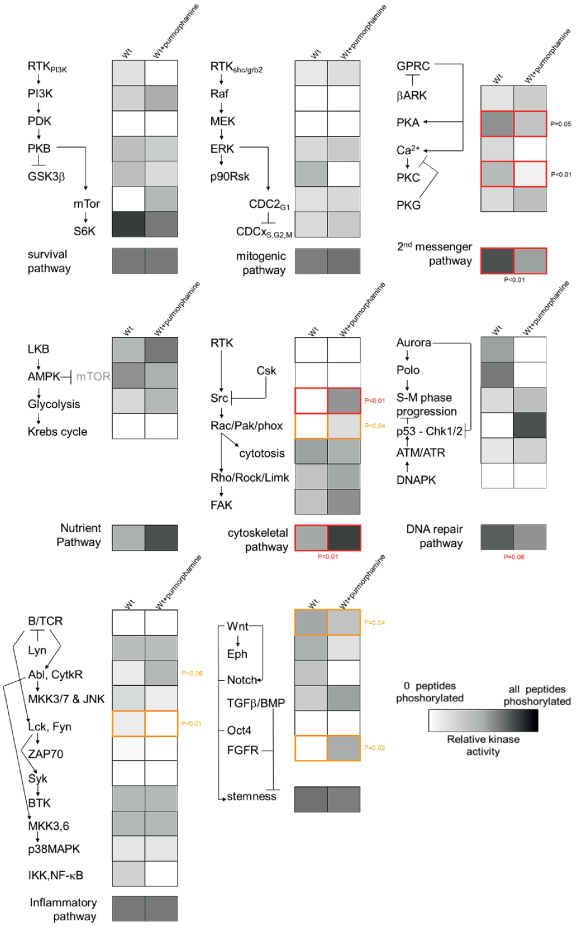

FIGURE 5. Effects of selective Smoothened activation by purmorphamine stimulation on cellular signaling in fibroblasts. Murine fibroblasts were stimulated with purmorphamine. Subsequently cells were lysed and the resulting lysates were used to phosphorylate arrays of different kinase substrates employing 33P-□ATP and radioactivity incorporated in the different substrates was determined. Peptide substrates were allotted to elective signal transduction elements. The picture depicts the number of peptides significantly phosphorylated (which means the number of peptides that received a Markov "on" call - see experimental procedures) for each element. A darker color reflects more kinase activity towards substrate elements and the results reveal the effects of purmorphamine stimulation on cellular signal transduction, thus a black color means all peptides were significantly phosphorylated, whereas a white color means that no peptides allotted to this signal transduction in this experimental conditions were phosphorylated. Results were first statistically tested by a dichotomal analysis based on the number of Markov "on" calls observed in vehicle-and Shh-stimulated cultures. If statistically significant differences were noted the signal transduction category is highlighted with a red border and the level of significance observed is indicated in red. For signal transduction elements in which this very robust analysis fails to detect a statistically significant difference, a parametric test was performed. If this proved significant, the category is highlighted with an orange color and corresponding level of significance is depicted as well – in orange. The results reveal a web of Smoothened-dependent signal transduction events clearly distinct from Patched-dependent signaling.

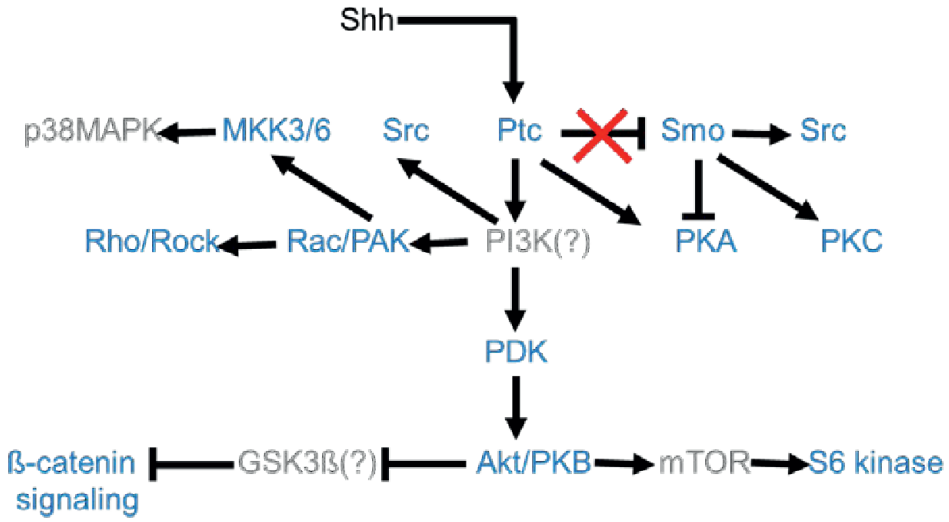

FIGURE 6. Selected kinome profiling-detected Shh-provoked signal transduction events and the role of Patched and Smoothened therein. Blue elements are confirmed, whereas gray elements showed a trend but did not reach Bonferoni-corrected statistical significance. The results reveal that the role of Patched dependent Smoothened-independent signal transduction is more prominent in transcription independent cellular effects of Hedgehog as previously thought.

Cellular kinase response to selective Smoothened activation

Subsequently we decided to investigate the effects of selective Smoothened activation in MEFs. To this end we employed purmorphamine, a purine derivative that directly targets Smoothened[45, 51] and employed the compound to challenge MEFs. The results are provided through Figure 4 and Supplementary table 1. We

observe that purmorphamine results in inhibition of PKA enzymatic activity, an effect which is also seen following stimulation of fibroblasts with Hedgehog and is thus dominant over the Smoothened-independent inhibition of its enzymatic activity. Intriguingly, purmorphamine results in a downregulation of Rho-associated protein kinase (ROCK). ROCK is important for a variety of cellular processes, but in particular for cytoskeletal reorganization[52]. It was earlier established that Smoothened is a powerful mediator of chemotactic responses, but only so when not located at the primary cilium[34]. At the primary cilium, Smoothened loses its capacity to stimulate chemotaxis. The apparent downregulation of ROCK activity following purmorphamine stimulation is thus best explained by a purmorphamine-dependent recruitment of Smoothened to the primary cilium. The strong canonical responses to purmorphamine stimulation observed by others would agree with this notion, as would the marked downregulation of PKA activity in our profiles. In some aspects, the rapid Smoothened-independent effects and rapid Smoothened-dependent effects on cellular kinase activities studied in our experimental set up, are similar, as both provoke mTOR activation and, in our model system, activation of Wnt signaling. In this sense, non-canonical signaling downstream of Patched and Smoothened may converge to produce the final phenotype. It is important to stress that our set up does not allow studying the effects of canonical Hedgehog signaling, which requires transcriptional responses. Generally speaking canonical signaling and non-canonical signaling by morphogens counteract each other and the effects observed in this study partially substantiate that notion for Hedgehog signaling as well. Not seen downstream of specific Smoothened stimulation were strong pro-inflammatory responses, which therefore seem mainly Patched-dependent. Generally speaking, Patched-specific signaling events (i.e. the effects of Hedgehog stimulation on Smoothened-/- fibroblasts) were less pronounced as those provoked by purmorphamine stimulation as also evident from the number of peptides that became significantly phosphorylated (see experimental procedures), in casu 180 peptides in Hedgehog-stimulated Smoothened-/- fibroblasts and 134 in purmorphamine-stimulated wild type fibroblasts. It thus appears that the major branch of non-canonical Hedgehog signaling is downstream of Patched but not of Smoothened.

Implications of Patched-dependent Smoothened-independent non-canonical Hedgehog signaling

Literature data indicate that the final effect of Hedgehog in physiology and pathophysiology is resultant from the integration of canonical and non-canonical Hedgehog signaling[29, 46]. Aberrant Hedgehog signaling is an important factor in human disease and accordingly inhibiting Hedgehog signaling has received substantial attention and various trials employing pharmacological inhibitors of Hedgehog signaling have been developed. Especially Vismodegib and Sonidegib have met with success in diseases driven by canonical Hedgehog signaling, in particular dermatological cancer[53]. Despite the evidence, however, that Hedgehog signaling is important for many gastrointestinal cancers[54], trials in this type of disease have not yet proven successful. In view of our data presented above that Patched and not Smoothened is a major mediator of non-canonical Hedgehog signaling and the momentum-gaining notion that especially non-canonical Hedgehog signaling may be important for maintaining gastrointestinal cancer[29], this may not be surprising. Vismodegib and Sonidegib target Hedgehog signaling at the level of Smoothened and leave Patched-dependent non-canonical Hedgehog signaling unaffected. Especially in view of the Patched-dependent Smoothened-independent Wnt signaling, one can easily imagine that especially the non-canonical branch of Hedgehog signaling is important in supporting growth in the gastrointestinal compartment. An implication of our results is thus that future Hedgehog-based therapy with respect to gastrointestinal cancer should be directed at counteracting the interaction of Patched with Hedgehog rather than the current strategy of targeting Smoothened.

Conclusion

Here we characterize the non-canonical aspect of Hedgehog signaling. We observe that such non-canonical signaling mainly involves Patched-dependent Smoothened-independent signaling, with especially mTOR signaling, the activation of cytoskeletal remodeling and the activation of Wnt signaling being prominent elements. Thus, for efficient targeting of Hedgehog-dependent signaling, it may prove essential to target such signaling at the level of Patched and not Smoothened.

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Author Contributions

Conceptualization, M.P.P., M.F.B., M.J.B and C.A.S; Methodology, K.P., and G.M.F; Investigation, A.I.A and L.B.; Writing – Original Draft , A.I.A.; Writing – Review & Editing, M.P.P., G.M.F., and M.F.B; Supervision M.P.P, and C.A.S.

Declaration Of Interests

The authors declare no competing interests.

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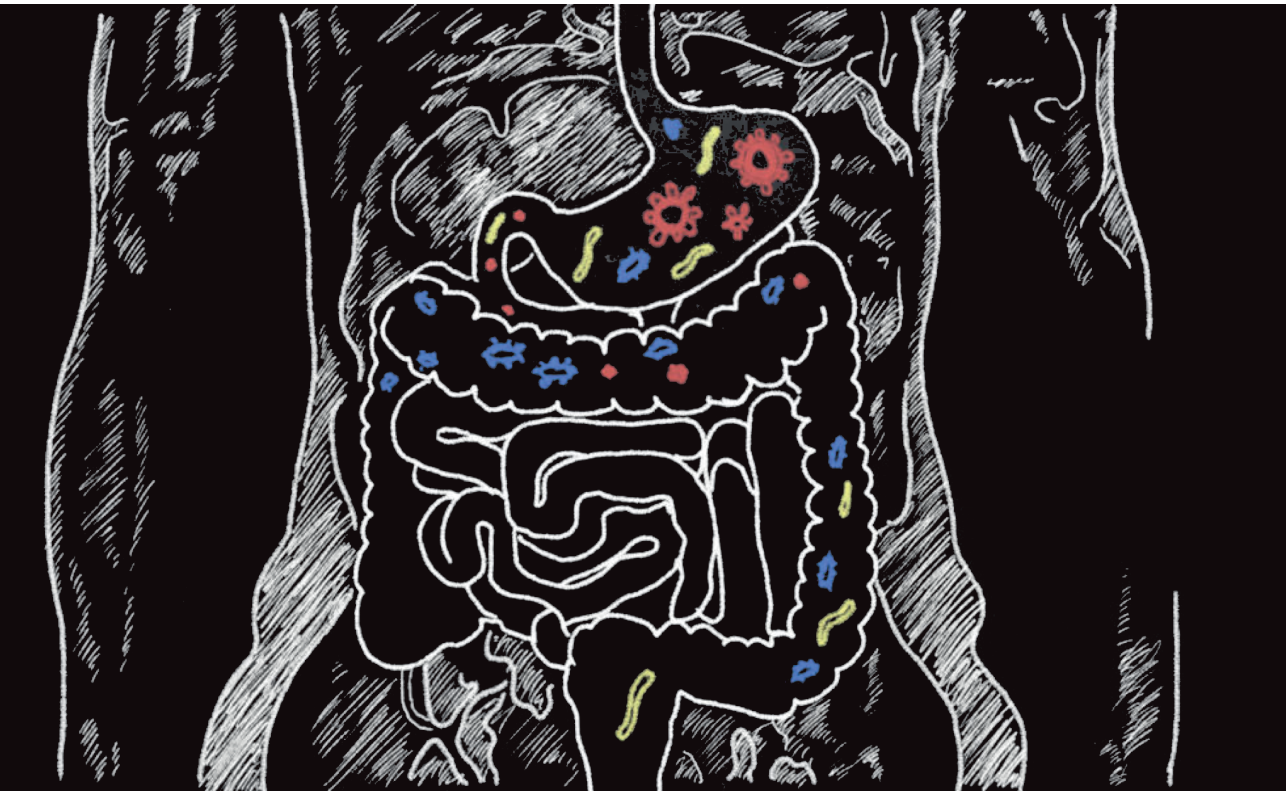

Chapter 5

Agreement Between Histologic And Endoscopic Helicobacter Pylori–Associated Intestinal Metaplasia And Gastric Atrophy

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Abstract

Atrophic gastritis (AG) and intestinal metaplasia (IM) are generally regarded as a precancerous condition associated with *Helicobacter pylori* and strongly predispose to the development of gastric cancer. Although the accurate diagnosis is essential for preventing gastric cancer, in practice many physicians rely solely on visual endoscopic inspection to rule out the presence of AG. It is thus important to verify the validity of such endoscopic inspection to the gold standard (histological diagnosis by the pathologist) in a variety of settings, also because incidence and presentation of AG is highly variable in geographical terms. This consideration prompted us to conduct a retrospective cohort study involving 248 patients of which, 124 are from low-incidence Nigeria and 124 from high incidence Iran, all aged 50±30 years. The extent of endoscopic atrophy was classified into five subgroups according to a modified Kimura–Takemoto classification system and was compared with histological findings of atrophy at five biopsy ,sites according to the updated Sydney system. The strength of agreement between endoscopic and histological atrophy was moderate and showed a substantial geographical discrepancy, indicating that relying solely on endoscopic screening for AG requires local validation.

Introduction

Gastric cancer (GC) remains the second most leading cause of cancer-related deaths and ranks 4th in cancer incidence worldwide[1, 2]. Incidence rates and presentation of gastric cancer show, however, marked regional differences, European countries tend to have a low incidence[3]. In contrast, in Iran for instance stomach cancer together with breast cancer has the highest incidence and highest mortality of all types of oncological disease in this country[4]. In contrast, gastric cancer has a low prevalence in sub- Saharan Africa with the lowest incidence rates in Western Africa[5]. Thus, gastric cancer is constitutes a global health issue, but presentation is markedly different in various parts of the world, raising questions as to whether screening strategies should to be tailored according to geography.

Appropriate screening is important as the prognosis of gastric cancer varies dramatically according to disease stage. The 5-year survival rate for advanced gastric cancer is less than 20%. In contrast, early gastric cancer (EGC) has a good prognosis, with reported 5-year survival rates being in excess of 90 % or even 95 %[5, 6]. The main risk factors for gastric cancer are Helicobacter pylori infection, salt intake, smoking, alcohol, a family history of gastric cancer, atrophic gastritis (AG), and intestinal metaplasia (IM)[7]. Especially AG and IM are important as they are considered to be premalignant lesions of gastric cancer[7, 8]. Hence accurate detection of AG and IM is essential for effective combat of gastric cancer.

AG and IM have multiple etiologies but the most important risk factor for these conditions is Helicobacter pylori infection[9-11] and according H. pylori eradication therapy provides a preventive effect with respect to gastric cancer development[12, 13]. Thus it is especially important to establish that screening for H. pylori-associated is adequate and appropriate irrespective of the geographical context. Currently the gold standard for the diagnosis of AG and IM is histological evaluation of biopsies by the pathologist[14]. In practice, however, in many cases physicians rely on endoscopic evaluation, especially for making a diagnosis of atrophy. Especially the endoscopic atrophy classification (EAC) according to Kimura and Takemoto[15, 16] is frequently been used to evaluate the atrophic degree of gastric mucosa. However, although intraobserver agreement for gastric mucosa atrophy using the Kimura-Takemoto Classification. tends good to excellent, interobserver agreement is moderate even in experienced endoscopists[17], suggesting that histology-free evaluation may be suboptimal. Surprisingly, however,

the correlation between endoscopic evaluation and histological final diagnosis has been relatively underexplored. Generally speaking published studies support that endoscopic evaluation performs well with respect to the detection of histological IM and AG[18, 19]. However, these studies were performed in specific east-Asian high risk cohorts and the extent as to which these results can be extrapolated to the global situation remains uncertain, Data in patients specifically following H. pylori eradication are not present at all. Thus there is paucity in studies assessing the accuracy of endoscopic detection of AG in different settings.

The above mentioned considerations prompted us to investigate the concordance between histological diagnosis and endoscopic diagnosis employing the Kimura-Takemoto Classification of AG in a variety of geographical settings. The results show that irrespective of the context endoscopic evaluation performs well.

MATERIALS AND METHODS

Study population

The study population consisted of a total of 248 (Nigeria-cohort: 124, Iran-cohort: 124) patients who underwent both upper gastrointestinal Zoom endoscopy and examination for detection of H. pylori. The Nigeria cohort was collected at the University of Abuja Teaching Hospital, Gwagwalada. Abuja. Nigeria and the Iran cohort at the Department of Medical Laboratory Sciences. Marand Branch, Islamic Azad University, Marand, Iran. Only patients presenting between January 2007 and August, 2017 were evaluated in this study. Exclusion criteria were as follows: patients with prior history of gastrectomy, endoscopic evidence of reflux esophagitis, peptic ulcer disease, or malignancy and patients who had been treated with antibiotics, proton-pump inhibitors, bismuth-containing compounds or histamine H2 receptor blockers within four weeks before the endoscopic procedure. Patients were also excluded if they had received H. pylori eradication therapy in the past or had been treated with any non-steroidal anti-inflammatory drug in the two weeks leading up to the endoscopic procedure. The protocol was approved by the Ethics Committee of the University of Abuja Teaching Hospital, Gwagwalada. Abuja. Nigeria and Department of Medical Laboratory Sciences. Marand Branch, Islamic Azad University, Marand, Iran. All patients gave written informed consent before entering the study. Two-hundred and forty-eight patients satisfied the criteria,

and systemic map biopsies were taken. In total, 131 men and 117 women; mean age, 46 years; range, 20–80 years were studied.

Histological examination

Records of biopsy sampling and the subsequent histological analysis by the gastroenterological pathologist were retrieved and reviewed. Biopsy samples used for the analysis were those obtained using standard biopsy forceps from the five sites specified in the updated Sydney system (Figure 1) and had to be processed according to convention procedures. With respect to the latter, each tissue sample included was placed in a separate bottle of 10% formalin and embedded in paraffin for sectioning. Sections were stained with hematoxylin and eosin (HE) and evaluated according to established procedures. All the specimens were scored by expert gastrointestinal pathologists. For analysis of specimens with IM, PAS and Alcian blue 2.5 staining had to be used to identify the IM subtypes. Gastric atrophy was defined as apparent chronic inflammation of the gastric mucosa with concomitant loss of the gastric glandular cells and their replacement by intestinal-like epithelium, pyloric-like glands, and fibrous tissue. In each single biopsy, atrophy was scored as a percentage of atrophic glands. Non-metaplastic and metaplastic atrophy were considered together. For each biopsy sample, atrophy was scored on a four-tiered scale (no atrophy = 0%, score = 0; mild atrophy = 1–30%, score = 1; moderate atrophy = 31–60%, score = 2; and severe atrophy >60%, score = 3). The OLGA stage resulted from the combination of the overall “antrum score” with the overall “corpus score” [20]. In each specimen, IM was subject to Markov classification (absent or present), and “extensive intestinal metaplasia” was deemed present if IM appeared in two or more specimens of the same patient. A patient was considered to have incomplete IM subtype if the incomplete subtype appeared in at least one specimen. Gastric dysplasia was assessed according the revised Vienna classification[21]. In this study, the expert gastrointestinal pathologists were blinded to the age and sex of the subjects. The graded features were scored according to the updated Sydney system for atrophy[22]. Patients were considered positive for histological atrophy if the score was mild, moderate or marked in each location.

All of the endoscopic examinations were performed and assessed by one experienced endoscopist (G.A and K.D) who had been trained to evaluate EGA at the University of Abuja Teaching Hospital, Gwagwalada. Abuja. Nigeria and Department of Medical Laboratory Sciences. Marand Branch, Islamic Azad University, Marand, Iran respectively. Olympus video-scopes with conventional white light (model GIF-160; Olympus) were used. The endoscopic mucosal atrophy was evaluated according to the location of the endoscopic atrophic border described by Kimura and Takemoto[17]. This atrophic border is the boundary between the pyloric and fundic gland regions, which is endoscopically recognized by the difference in color and height of the gastric mucosa between the two sides of the border (Fig. 1). There are three grades of EGA: severe (O2 – O3), moderate (C3 – O1), and mild (C1 – C2). Six specimens were taken from each patient: five specimens were taken from specific locations according to the updated Sydney System and were put in separate boxes for pathologic examination; the 6th specimen used for rapid urease test was taken from the greater curvature of the antrum.

Level of agreement definition

The endoscopic findings of the extent of atrophy were compared with the histological findings of glandular atrophy at five biopsy sites (Figure 1). To be able to compare the extent of atrophy strictly, both classifications were modified to five grades according to definitions of those anatomical locations. Histological grading was scored as 1, none; 2, antrum (site 1 and/or 2); 3, angulus (up to site 3); 4, the middle body of the lesser curvature (up to site 4) and 5, the middle body of the greater curvature (up to site 5). Endoscopic atrophic grading according to the modified Kimura–Takemoto classification was scored as 1, none; 2; antral (C-1); 3, antral predominant (C-2); 4, corpus predominant (C-3, O-1, O-2) and 5, pan-atrophy (O-3) (Figure 3). Inasmuch as extensive atrophy is associated with a much higher cancer risk than limited atrophy, the Kimura–Takemoto classification was simplified to three grades of cancer risk oriented atrophy as: normal (no atrophy), limited atrophy (antral and antral predominant atrophy; C-1, C-2) and extended atrophy (corpus predominant and pan-atrophy; C-3, O-1, O-2, O-3). Agreement was defined as matching of endoscopic and histologic grades, with all other findings defined as disagreement.

Serum pepsinogen levels

Fasting serum was collected from all subjects. The samples were centrifuged immediately at 4 °C and serum stored at −70 °C until used. Serum concentrations of pepsinogen (PG) I and II were measured using a latex-enhanced turbidimetric immunoassay, and the PG I to PG II ratios (PG I/II) were calculated.

***Helicobacter pylori* detection.**

Serum samples from all patients were tested by enzyme linked immunosorbent assay for the presence and concentration of IgG antibodies to *H. pylori* (HM-CAP; Enteric Products Inc., Westbury, NY). A concentration ≥1.8 was defined as positive (sensitivity 98.7%, specificity 100%).We also employ the gold standard for H. pylori detection by culture. Following collection of gastric biopsy, samples were homogenized and cultured onto Brucella agar supplanted with 5% sheep blood and antibiotics (Vancomycin, Amphotericin B and Trimethoprim). Culture plates were incubated at microaerophilic condition, 37 ºC and high humidity for 5-7 days. Organisms were identified as H. pylori based on colony morphology, gram staining and positive oxidase, catalase and urease tests.

Statistical analysis

STATA software (version 10.0; Stata Corp, College Station, TX, USA) was used. Chi-squared test and Fisher's exact test were applied to evaluate endoscopic, histological, and serological parameters in patients with gastric atrophy. Agreement between endoscopic and histologic evaluations of the grade of gastric atrophy was assessed by determining the weighted kappa value. Factors associated with extensive atrophy were estimated by univariate logistic regression analysis. Covariates showing a significant association with extensive atrophy by the χ^2 or *t* test were included in multiple logistic regression analyses. Odd ratios (ORs) and 95% confidence interval (CI) were calculated to assess the strength of association between variables. A P value < 0.05 was considered statistically significant.

RESULTS

Demographic data

A total of 248 patients aged 50±30 years were included in this study, including 124 patients from Nigeria and 124 from Iran. Mean ± SD patient age was 46.4 (± 15.3) years. Of these patients, 131(52.8%) were male, 117 female;(47.2%). Gender ratio F/M :0.89 and 138 (55.6%) were serologically *H. pylori-positive*. The detailed characteristics of the two subgroups are shown in **Table 1**. There were significant differences between the groups from the Nigeria and Iran, especially with respect to the extent of atrophy. In the Nigerian population, only 60.% were diagnosed histologically of having corpus atrophy, whereas, in Iran, 54% had gastric atrophy while 57 (46.0%) showed no evidence of histological atrophy. Hence geographical origin influences disease presentation necessitating comparison of the relative performance of endoscopy with respect to the diagnosis of AG.

Table 1: Demographic, endoscopic, and pathologic characteristics of study population with *H pylori* associated gastric atrophy.

	Nigeria (n = 124) (%)	Iran (n =124) (%)	Total (n =248) (%)	P value (Nigeria vs Iran)
Clinical parameters				
Sex				
Male	62 (50.0)	69 (55.6)	131 (52.8)	0.37
Female	62 (50.0)	55 (44.4)	117 (47.2)	
Age (yr)				
≥ 40	69 (55.6)	68 (54.8)	137 (55.2)	0.89
< 40	55 (44.4)	56 (45.2)	111 (44.8)	
Helicobacter pylori Ag				
Positive	40 (32.3)	88 (71.0)	128 (51.6)	0.84
Negative	84 (67.7)	36 (29.0)	120 (48.0)	
Helicobacter pylori IgG				
Positive	89 (71.8)	49 (39.5)	138 (55.6)	0.00
Negative	35 (28.2)	75 (60.5)	112 (45.4)	
Serologic features (ng/mL), mean ± SD				
Pepsinogen I	49.9 ± 39.2	57.8 ± 38.1	53.1 ± 38.7	0.731
Pepsinogen II	12.9 ± 7.2	12.1 ± 10.1	12.9 ± 8.65	0.621

Pepsinogen I/II ratio	5.4 ± 1.8	<5		0.471
Endoscopic atrophy				
No atrophy	50 (40.3)	67 (54.0)	117 (47.2)	0.00
Antral (C-1)	43 (34.7)	36 (29.2)	79 (31.9)	
Antral predominant (C-2)	26 (20.9)	0 (0.0)	48 (19.4)	
Corpus predominant (C-3-O-2)	5 (4.03)	21 (17.0)	26 (10.5)	
Histological atrophy				
None	50 (40.3)	57 (46.0)	107 (43.1)	0.00
Antrum	38 (30.2)	41 (33.0)	79 (31.8)	
Angulus	20 (16.13)	26 (20.5)	46 (18.5)	
Lesser curvature of middle body	16 (12.9)	0 (00.0)	16 (6.5)	
Greater curvature of middle body	0 (0.0)	0 (00.0)	0 (0.0)	
Intestinal Metaplasia	85 (68.5)	63 (50.8)	148 (59.7)	0.06
Complete subtype	46 (37.1)	32 (25.8)	78 (31.4)	
Incomplete subtype	30 (24.2)	26 (20.9)	56 (22.5)	
Unidentified	9 (7.2)	5 (4.1)	14 (5.6)	

Agreement between endoscopic and histological atrophy

The comparisons between endoscopic and histological atrophy scores are shown in **table 2**. Taking the study population *in toto,* of the 248 patients, 138 (55.0%) showed complete agreement between endoscopic assessment and final histological diagnosis. Importantly, the strength of agreement between the modified Kimura–Takemoto classification and histological atrophy, as defined by the updated Sydney system, showed good reproducibility, with a weighted kappa value of 0.89 [95% confidence interval (CI) 0.68–0.96]. However, a total of 110 patients were endoscopically misdiagnosed, including 45 (18.14%) who were over-diagnosed and 65 (26.2%) who were under-diagnosed. Of the 43 patients histologically diagnosed with atrophy in the middle of the body of the greater curvature, 21 (49 %) were under-diagnosed endoscopically. Moreover, 30 of 110 (127.3%) patients without histological atrophy were endoscopically over-diagnosed with antral or antral predominated atrophy. Thus generally speaking endoscopic assessment performs well but is not sufficient for accurate diagnosis

		Histological atrophy					Total	Weighted κ value
		None	Antrum	Angulus	Middle body LC	Middle body GC		
Endoscopic atrophy	No atrophy	**71**	21	14	11	0	117	0.89
	Antrum (C-1)	18	**42**	9	10	0	79	
	Antrum predominant (C-2)	0	2	**3**	0	0	5	
	Corpus predominant (C-3-O-2)	12	3	10	**22**	0	47	
	Pan-atrophy(O-3)	0	0	0	0	**0**	0	
	Total	101	68	36	43	0	248	
	Misdiagnosis	30	26	33	21	0	110 (44.35%)	
	Concordance		Endoscopic over-diagnosis			Endoscopic under-diagnosis		

Factors affecting Agreement

To identify factors affecting the agreement between endoscopic and histological atrophy, univariate analyses were performed; factors analyzed included geopgraphy (Nigeria vs Iran), age, sex, *H. pylori* infection, endoscopic atrophy (no atrophy *vs* others) and PG I/II ratios. Factors significantly associated with reduced performance of endoscopy included Iran ethnicity ($P < 0.001$), older age ($P < 0.001$), a low pepsinogen I/II ratio ($P = 0.015$), the endoscopic dwetection of atrophy ($P < 0.001$) and *H. pylori* infection ($P = 0.001$) (**Table 3**). In contrast, age was not significantly associated with reduced Agreement ($P = 0.138$). Multivariate analysis showed that only three factors were independently associated with reduced agreement: Iranian ethnicity, older age and endoscopic atrophy (**Table 4**). Thus the performance of endoscopic evaluation is influenced by the geographical context.

Table 3: Factors that significantly associated with reduced concordance between endoscopic and histological atrophy with H pylori Associated gastric atrophy cases.

	Agreement group (*n* = 180) (%)	Disagreement group (*n* = 58) (%)	OR(95%CI)	P value
Country				
Nigeria	98 (51.6)	38 (65.5)	0.22 (0.23 -0.76)	<0.001
Iran	92 (48.4)	20 (34.5)	1	
Age (yr)				
≥ 40	109 (57.4)	30 (51.7)	0.14 (0.09 -0.40)	P=0.148
< 40	71 (42.6)	28 (48.3)	1	
Helicobacter pylori IgG				
Positive	111 (58.2)	32 (55.2)	0.40 (0.30 -0.55)	P=0.001
Negative	69 (36.3)	26 (44.8)	1	
Serologic features (ng/mL)				
Pepsinogen I	120 (63.2)	38 (65.5)	0.23 (0.20 -0.45)	P=0.001
Pepsinogen II	70 (36.8)	20 (34.5)	1	
Endoscopic atrophy				
No atrophy	89 (46.8)	22 (37.9)	1	
Others	101 (53.2)	36 (62.1)	0.38 (0.22 -0.67)	<0.001

Further assessments according to factors significant on multivariate analysis

To further assess the disagreement between histological and endoscopic atrophy, patients were cross-tabulated by each factor found to be significant (**Table 4**). Although the performance of endoscopic evaluation was diminished in extensive atrophy in both populations, the geographical context influenced the extent of this misdiagnosis. More specifically, 24 (19.35%) of the 124 Nigeria patients were misdiagnosed, including 15 who were over-diagnosed with histological atrophy of the antrum or angulus. In contrast, 57 (45.2%) of the 126 Iranian patients, of all histological grades, were misdiagnosed, including 22 who were over-diagnosed and 35 who were under-diagnosed. This further analysis supports the notion that the performance of endoscopic evaluation solely is significantly influence by the different presentation of disease in alternative geographical locations.

Table 4. Factors that significantly associated with reduced concordance between endoscopic and histological atrophy with H pylori-associated gastric atrophy cases.

	Adjusted OR (95%CI)	P values
Country		
Nigeria	1	<0.001
Iran	0.23 (0.11–0.56)	
Age (yr)		
≥ 40	1	0.268
< 40	0.6 (0.31–1.38)	
***Helicobacter pylori* IgG**		
Positive	1	
Negative	0.32 (0.16–0.66)	0.008
Pepsinogen I/II ratio		
> 3.0	1	
≤ 3.0	0.50 (0.67–3.35)	0.328
Endoscopic atrophy		
No atrophy	1	0.256
Others	0.09 (0.04–0.56)	

Cancer risk-oriented atrophy grading

When classified according to cancer risk-oriented atrophy grading defined above, 110 (88.1%) of the 248 patients were concordant (**Tables 5 and 6**). The strength of agreement between endoscopic and histological assessment by cancer risk-oriented grading showed good reproducibility, with a weighted kappa value of 0.79 (95%CI: 0.60–0.97). No marked differences between the two geographical locations involved appeared. Thus with respect to assessing cancer risk endoscopic evaluation performs well irrespective from the geographical context.

Table 5. Endoscopic and histological atrophy cross tabulation according to populations with *H pylori* associated gastric atrophy

		Histological atrophy						
Nigeria		**None**	**Antrum**	**Angulus**	**Middle body LC**	**Middle body GC**	**Total**	**Weighted κ value**
Endoscopic atrophy	No atrophy	**40**	4	4	2	0	56	0.79
	Antrum	8	**30**	5	**0**	0	40	
	Antrum predominant	0	2	**3**	0	0	24	
	Corpus predominant	4	0	10	**12**	0	6	
	Total	52	36	22	14	0	124	
Iran								
Endoscopic atrophy	No atrophy	**31**	17	10	09	0	67	0.96
	Antrum	10	**12**	4	**10**	0	36	
	Antrum predominant	0	0	**0**	0	0	0	
	Corpus predominant	8	3	0	**10**	0	21	
	Total	49	32	14	29	0	124	

Table 6: Risk factors-associated with cancer

		Histological atrophy			Total	Weighted κ value
		Normal	Limited	Extended		
Endoscopic atrophy	Normal	**70**	5		75	0.79
	Limited (C-1~2)	24	**80**	7	107	
	Extended (C-3~O-3)		12	**52**	62	
	Total	92	97	59	248	
	concordance		endoscopic over-diagnosis			endoscopic under-diagnosis

Association between OLGA Gastritis Stage, Endoscopic gastric atropy and Intestinal Metaplasia

Intestinal metaplasia, extensive IM, and incomplete IM subtype also clustered to the subgroup of patients with high-stage OLGA gastritis (**Table 8**).

Table 7. Association between endoscopic gastric atrophy and metaplasia

	Endoscopic gastric atrophy			
	Moderate-to severe (n = 40)	**None-to-mild (n =38)**	**P (X^2 test)**	**Adjusted Odd ratios (95% CI)**
Complete IM	27.5% (11/40)	47.34% (18/38)	**<0.001**	21.3 (8.7–35.2)
Incomplete	30.0% (12/40)	31.6% (12/38)	**<0.001**	33.5 (7.4–37.3)
Unidentified	42.5 % (17/40)	36.84(14/38)	**<0.001**	41.7 (4.1–74.1)

IM, intestinal metaplasia

Table 8. The association between OLGA gastritis stage and intestinal metaplasia

	OLGA gastritis stage			
	Stage 0–II (n = 121)	**Stage III, IV (n = 47)**	**p (Fisher's exact)**	**Odd ratios (95% CI)**
IM	49.58 (60/121)	38.29 (18/47)	**<.001**	**24.6 (05.7–72.5)**
Extensive	26.45 (32/121)	25.53 (12/47)	**<.001**	**22.6 (07.8–82.9)**
Incomplete subtype	18.18 (22/121)	21.27 (10/47)	**<.001**	**33.7 (04.5–62.8)**
Unidentified subtype	4.13 (7/121)	0 (0/47)		

Seven cases with unidentified IM subtype were all in OLGA gastritis stage 0–II. When we subsequently analyzed only patients with gastritis stage 0–II only, there was no homogenous distribution of IM subgroup: We rarely found patients with IM none-to-mild EGA, while it clustered to patients with moderate-to-severe endoscopic gastric atrophy (**Table 7**). We also found that there was an association between the gastric atrophy and the subtype of IM. From a total of 59.7% (148 /248) in our entire study population, 46(37.1%) and 32(25.8%) from Nigeria and Iran respectively had the complete subtype of IM while 30 (24.2%) and 26 (20.9%) patients from Nigeria and Iran respectively presented with the incomplete IM. In addition, 9 (7.2%) and 5 (4.1%) patients from Nigeria and Iran respectively were classified as indeterminate IM. Thus also with respect to the presentation of IM, important regional differences are apparent, further highlighting the necessity to tailor screening strategies to local needs.

Discussion.

The natural history of the development and progression of gastric cancer in general and especially the role of H. pylori infection in this process is now fairly well understood and involves sequence of gastric mucosal atrophy, intestinal metaplasia, and gastric cancer[23]. This has led to the realization that endoscopic screening in high-risk individuals is essential for preventing the associated mortality. The efficacy of such screening obviously depends on adequate diagnosis in which histological assessment of biopsies taken by the endoscopist remains the gold standard. The updated Sydney system was designed to assess histological gastritis and atrophy more objectively and has become the international standard[24]. Although this classification includes assessment of five biopsy sites, this extensive

approach is not common in daily practice, because of patient discomfort, cost, logistic and time restrictions. Hence, many physicians rely to an important extent on endoscopic evaluation for the detection of AG. To which extent this is a problem is not well understood. A Swedish study found a sensitivity and specificity for moderate to severe atrophic gastritis in the gastric corpus of 67 % and 85 %, respectively[25] and concluded that macroscopic features as observed during gastroscopy are of very limited value in the evaluation of whether or not gastritis or H. pylori infection are present. A Korean study, however, reported that endoscopic and histological atrophic gastritis show relatively good correlations[16, 25]Hence further studies, investigating presentation in different geographical contexts are necessary to clarify to which extent endoscopic observation alone can accurately assess AG. The present study was initiated to fill this void.

Our results show that the efficacy of endoscopy to detect AG is moderate and shows substantial regional variation, possibly caused by different presentation and incidence of AG at different locations around the globe. Generally speaking the performance of endoscopic screening is not good enough to rely on it alone and histological confirmation of the endoscopic diagnosis remains necessary. In conjunction with geographical variations in the performance of endoscopic observation to detect AG, it is fair to say that local validation remains essential. The agreement rate for atrophy was significantly higher for patients in the Nigerian than for those in Iran. Much more patients, however, in the Iranian cohort displayed extended atrophy, which is more easily misdiagnosed. The two populations also had different concordance between endoscopic diagnosis and microscopic diagnosis in the multivariate analysis, suggesting that this difference may be associated with differences in the background of the two populations. Indeed, the two populations differed in host genetic factors, diet, and bacterial virulence. For example, there are ethnic and.or geographical differences in the H. pylori cytotoxin associated gene A (CagA), one of the most important virulence factors for gastric mucosal injury and atrophy. The CagA gene is polymorphic and is primarily classified into East Asian and Western types based on sequences in its 3′ coding region[10]. Previous studies have clarified the differences in gastritis and atrophy among patients infected with East Asian CagA-positive, Western CagA-positive, and CagA-negative H. pylori[26]], with differences in virulence ppotentially provoking differences in agreement rates. In Iran the cagA gene genotype was found to predominate in gastric adenocarcinoma patients[27]. But obviously further studies, linking such variations

directly to concordance between endoscopic and histological diagnosis are necessary to substantiate this notion.

We observed that older age predisposes for discordance between the endoscopic assessment and the histological diagnosis, The histological structures of the normal antral and corpus mucosa differ, with the border between these two areas located at the angulus. This anatomically defined border is difficult to detect clearly using conventional endoscopy[27, 28][27, 28][27, 28] although it can be better detected using high definition equipment. However, a slight difference in color between the antrum and the corpus may occur in the absence of histological atrophy. Mistaking this anatomical border for the atrophic border may result in over-diagnosis. We found that older patients, particularly older Iranians, tended to be over-diagnosed. Two possibilities may explain this finding. The first may have been bias in the endoscopist prompted by the notion that elderly Iranians are more likely to be infected with H. pylori. The second reason is that gastric mucosal blood flow decreases with age[29, 30], which may affect mucosal appearance. Because of the slight differences in mucosal color, the endoscopist may mistake normal for atrophic antral mucosa in older patients. It is clear, however, that special care should be taken in older patients and that the practitioner should not submit to the temptation to rely solely on endoscopic diagnosis.

Conclusion

Although routine endoscopy can assess precancerous conditions by evaluating the extent of gastric atrophy, agreement between endoscopic and histological atrophy is unclear and always requires local validation. Endoscopic atrophy grading can predict extensive histological atrophy and may serve as a practical assessment of precancerous conditions during endoscopy in routine clinical practice, especially for patients in Western countries.

Competing interests

The authors declare that they have no competing interests.

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Nigeria.

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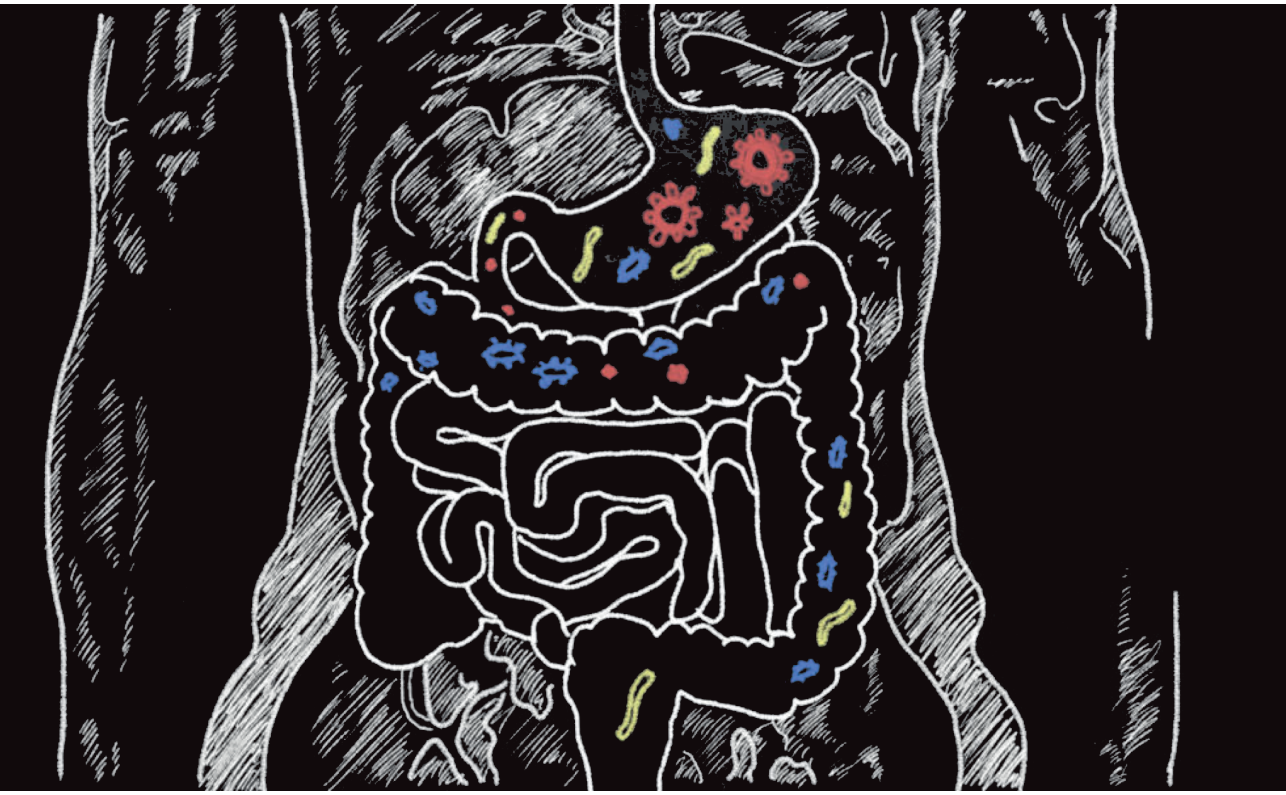

Chapter 6

Helicobacter pylori pathogenicity factors–related to gastric cancer

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Abstract

Background: Although the causal relationship between *Helicobacter pylori*-infection and the development of gastric cancer is firmly established, the exact nature of the virulence factors of *H. pylori* that predispose to gastric oncogenesis remains incompletely characterized. Such knowledge would aid stratification of *H. pylori*-infected individuals with respect to their risk to GC. We decided to investigate the association between *H.pylori* virulence genotypes and disease in a well-characterised cohort consisting of 109 *H.pylori* isolates from gastric biopsies originating from patients. **Methods:** The prevalence of genotype was assessed by PCR and related to clinical histopathological parameters. **Results:** We were able to identify association between genotypes and clinical outcome. The relation of *bab***A2**, *bab***B** negative and *ice***A1** positive genotype as a single genotype and the development of cases to GC (**S**ignet **R**ing **C**ell **C**arcinoma) was statistically significant (P <0.001).The *cag***E,** *cag***A,** *ice***A1** were found more commonly in patients with GC as compared with the other patient groups. The relation of the presence of *ice***A1** and the development of cases to GC was statistically significant (*P*=0.008), but *bab***A2**, *bab***B** alleles were not detected in these GC patients. These apparent negative association were still statically significant (*P*=0&0.005). **Conclusion:** Our results show an elevated prevalence of infection with *H. pylori* strains carrying known virulence genotypes with high genetic diversity. This highlights the importance of identifying gene variants for an early detection of virulent genotypes. Also, the results identify *H. pylori* genotypes associated with clinical outcome that may become potentially useful for patient risk stratification for GC.

Keywords: *Helicobacter pylori*, pathogenicity factors, gastric cancer

1. Introduction

Gastric cancer remains a prevalent disease worldwide with a poor prognosis. Helicobacter pylori plays a major role in gastric carcinogenesis. H. pylori colonization leads to chronic gastritis, which predisposes to atrophic gastritis, intestinal metaplasia, dysplasia, and eventually gastric cancer. Screening, treatment, and prevention of H. pylori colonization can reduce the incidence of gastric cancer[1]. Other interventions that may yield a similar effect, although of smaller magnitude, include promotion of a healthy lifestyle including dietary measures, non-smoking, low alcohol intake, and sufficient physical activity[2]. Furthermore, increasing evidence suggests that host factors, including genetic make-up, are also important determinants for carcinogenesis in H. pylori infection[3]. Colonization of H. pylori has been associated with chronic gastritis because it can trigger fulminant inflammatory response which can lead to pathological conditions such as Gastric carcinoma and peptic ulcer disease[4]. Consequently a clinical need exists to stratify H. pylori-infected patients with respect to their propensity to develop H. pylori-related pathology, especially for GC this is felt as a pressing concern. The importance of H. pylori virulence factors is evident from the serious clinical outcome associated with bacteria positive for the vacillating cytotoxic (vacA) and the cytotoxin-associated gene A (cagA) antigen[5]. However, these two virulence factors are insufficient to explain the variety in clinical presentation of pathology associates with H. pylori infection[6, 7]. A potential H. pylori virulence factor possibly important to explain this variance in clinical outcome is the cag pathogenicity island (cag-PAI). The cagA gene is a marker for the presence of the cag-PAI of approximately 40 kb, whose presence is associated with the more severe clinical outcomes.[8, 9] A type IV secretion system translocate cagA protein into gastric epithelial cells, where it is phosphorylated. When this modification occurs, cagA affects various cellular processes and signal transduction pathways, such as disruption of tight and adherent junctions that lead to pro-inflammatory and mitogenic responses–effects [8, 10]. One of the six cag-PAI genes is cagE, located in the right half of the cag-PAI, that has been shown to induce secretion of interleukin (IL)-8, from infected host epithelial cells[11, 12].

Another putative virulence factor is iceA, whose gene has two main allelic variants, iceA1 and iceA2. The expression of iceA1 is up-regulated on contact of H. pylori with human epithelial cells and may be linked with peptic ulcer disease.[13, 14] The blood group antigen binding adhesin (babA), a 78-KDa outer membrane protein

encoded by the babA2 gene, binds to Lewis b antigens and ABO antigen[15, 16] Although three bab alleles have been identified (babA1, babA2, and babB), only the babA2 gene product is functionally active[17] Studies in Western populations have associated the presence of the babA2 gene with gastric cancer[16, 18]. The aim of this studies is to assess the genotype of H. pylori strains infecting patients with chronic gastritis through the evaluation of the prevalence of several genes coding for virulence factors.

Materials and Methods

This research was approved by the regional Medical Research Ethics Committee of Azad University of Medical Science on 19 Jul 2016 (No: 1311/28772) and all patients provided written informed consent for this research.

This study protocol conforms to the ethical guidelines of the 1975 Declaration of Helsinki as Reflected in a prior approval by the institution's human research committee.

Patients, Bacterial strains and cultivation

Our cohort consisted of 109 clinical isolates of *H. pylori,* 49 (45%) were from female and 60 (55%)

From male patients (sex ratio F/M: 0.82); the average age of the patients from which the isolates were obtained was 39 ±17 years. *H. pylori* was isolated employing gastric biopsies of patients presenting with gastritis, peptic ulcer or gastric cancer. Patients were not exposed to antimicrobial agents at least one week before endoscopy was performed. Following collection, gastric biopsy samples were homogenized and cultured onto Brucella agar supplanted with 5% sheep blood and antibiotics (Vancomycin, Amphotericin B and Trimethoprim). Culture plates were incubated at microaerophilic condition, 37 ºC and high humidity for 5-7 days. Organisms were identified as *H. pylori* based on colony morphology, gram staining and positive oxidase, catalase and urease tests.

DNA extraction from culture

Genomic DNA of total *H. pylori* isolates was extracted using the QIAmp DNA mini kit (QIAGEN, Hilden, Germany) according to manufacturer's protocol and stored at -20 $^{\circ C}$ until use.

Detection of genes

In this study, PCR was used to detect the H. pylori specific ureC gene for confirming that the cultures represented a bona-fide H. pylori isolate, and the same technique was employed to establish presence or absence of cagE, cagA, iceA1, iceA2 babA2 and babB genes.

All primer sets were selected from published literature (listed in Table 1). PCR reactions were performed in a volume of 50 µL containing 10 mmol/L Tris-HCl, 1.5 mmol/L MgCl2, 0.2 mmol/L of each deoxynucleotide, 25 pmol of each primer and 2.5 units of Taq polymerase (Geneone, Germany)[19, 20]. The thermal cycler program used consisted of the following steps; initial denaturation at 94ºC for 3 min followed by 35 cycles of 30 seconds at 94ºC (denaturation), 30 seconds at 58ºC for cagA and glmM [21]; 30 seconds at 53ºC for cagE; 48°C for babA2, 49°C for babB, 57ºC For iceA1 and 48ºC and for iceA2 all annealing steps, followed by 30 seconds at 72ºC (extension step) and a final extension step was 3 min at 72 ºC[22].

Table 1: Primer sequences for polymerase chain reaction

Gene	Primer	Nucleotide sequence	Size (bp)	Ref
*ure***C** (*glm***M**)	Hp-F HP-R	GGATAAGCTTTTAGGGGTGTTAGGGG GCTTACTTTCTAACACTAACGCGC	294	(84)
*cag***E**	*cag***E**-F *cag***E**-R	TTGAAAACTTCAAGGATAGGATAGAGC GCCTAGCGTAATATCACCATTACCC	508	(85)
*bab***A2**	*bab***A2**-F *bab***A2**-R	CCAAACGAAACAAAAAGCGT GCTTGTGTAAAAGCCGTCGT	271	(6)
*bab***B**	*bab***B** -F *bab***B**-R	ATGAAAAAAACCCTTTTAC CGAATTGCAAGTGATGGT	496	(86)
*cag***A**	*cag***A**-F *cag***A**-R	AGGGATAACAGGCAAGCTTTTGA CTGCAAAAGATTGTTTGGCAGA	352	(87)
*ice***A1**	*ice***A1**-F *ice***A1**-R	GTGTTTTTAACCAAAGTATC CTATAGCCASTYTCTTTGCA	247	(84)
*ice***A2**	*ice***A2**-F *ice***A2**-R	GTTGGGTATATCACAATTTAT TTRCCCTATTTTCTAGTAGGT	229	(84)

Statistics analysis

Data were analysed by SPSS version 16. The Fisher's exact test or the Chi-square test was used for analysis of categorical data. A *p*-value of <0.05 was considered statistically significant.

Results

Description of the patient cohort and isolation of study samples

A total of 109 *H. pylori* samples were isolated from an initial number of 359 biopsies included in this study (yielding an apparent infection rate of 30.4%), of which 60 (55%) were derived from male patients and 49 (45%) isolates came from female patients (sex ratio F/M: 0.82). The average patients' age at the time of endoscopy was 39 ±17 years. From the 109 patients with established *H. pylori* infection, 81 patients had presented with a non-ulcer dyspepsia, nineteen patients with a peptic ulcer dyspepsia and nine patients with GC (Signet Ring Cell

Carcinoma (SRCC). There was no significant difference between the mean age and sex of patients with and without ulcers and cancer. We concluded that our cohort would enable analysis of the link between *H. pylori* virulence factors and clinical presentation.

The presence of virulence factors is common in the clinical cohort

The PCR-based amplification showed that the *cag***E,** *bab***A2** and *bab***B** positive strains had a prevalence of respectively 55.9%, 71.7%, 61.3% in our cohort, whereas *cag***A**, *ice***A1** and *ice***A2** were detected in respectively 70.6%, 42.2%, 13.2% of the patients included in this study (**Table 2**). Thus, the virulence factors selected for this study are commonly detected in our patient cohort and allow statistical analysis as to their relation to disease manifestation.

Specific *H. pylori* virulence factors show trends of being positively or negatively associated with patient's clinical presentation

In our study the frequency of *cag***E**-positive isolates obtained from patients with non-ulcer dyspepsia, peptic ulcer dyspepsia or GC patients was 49.4%, 68.4% and 88.9%, respectively, but this apparent positive association with more severe disease did not reach statistical significance ($P = 0.198$). The *bab***A2** genotype was detected in 74%, 84.2%, and 0% of isolates from NUD, PUD and GC patients, The percentage of *bab***A2**$^{-}$ genotype within GC patients was significantly higher than that of *cag***A**$^{+}$ genotype ($P <0.001$).

Also the percentage of *bab***B**$^{+}$ were 65.4% and 63.1% for NUD and PUD cases respective ,but for isolates from patients with GC (0%), this apparent negative association was statically significant ($P = 0.005$). Conversely the prevalence of the *ice***A2** allele was observed in NUD patients (13.6%) and in PUD (15.8%) and in GC patients (0%), but No significant association was observed between *ice*A2 genotype and GC (P = 0.779). Similarly, in this study the distribution of *ice***A1** and clinical outcome was analyzed statistically and it was observed that the frequency of *ice***A1**-positive isolates in NUD, PUD and GC patients was 35.8% and 42.1% and 100%, respectively. The relation of the presence of *ice***A1** and the development of cases to gastric cancer was statistically significant ($P =0.008$).

Although, the *cag***A** allele was observed in NUD (65.4%) cases and in PUD patients (78.9%) and in GC patients (100%), there was not statistically significant association between *cag***A** and the gastric outcomes (P =0.134). A full overview of all virulence factors studied and their linkage to specific clinical manifestations is provided through **Table 2**.

Table2: Relationship between clinical outcome and status of *cag*E, *bab*A2, *bab*B, *ice*A1, *ice*A2, *Cag*A (NUD: Non-ulcer dyspepsia, PUD: Peptic ulcer disease, GC: gastric cancer, *P*v: p-value)

Genotypes	Number (%) of isolates			Total (n=109)	*p-value*
	NUD (n=81)	PUD (n=19)	GC (n=9)		
*cag***E**	40 (49.4%)	13 (68.4%)	8 (88.9%)	61 (55.9%)	0.198
*bab***A2**	60 (74%)	16 (84.2%)	0	76 (71.7%)	0.001
*bab***B**	53 (65.4%)	12 (63.1%)	0	65 (61.3%)	0.005
*ice***A1**	29 (35.8%)	8 (42.1%)	9 (100%)	46 (42.2%)	0.008
*ice***A2**	11 (13.6%)	3 (15.8%)	0	14 (13.2%)	0.779
*cag***A**	53 (65.4%)	15 (78.9%)	9 (100%)	77 (70.6%)	0.134

It is apparent from our data that, *cag***E**, *cag***A** and *ice***A1** are more common in patients with gastric cancer than in the other patient groups, whereas *bab***A2** and *bab***B** alleles were absent in patients with GC.**Combining different virulence factors allow stratification of the patient cohort with respect to patient stratification**

We examined eight different combinations based on analysis of *bab***A2**, *bab***B** and *ice***A1** genotypes (positive and negative) in patients as a single genotype (Table 3). We were able to identify an association between these genotypes and clinical outcome. The frequency distributions of the combination genotypes of *H. pylori* showed the relation of *bab***A2**, *bab***B** negative and *ice***A1** positive genotype and the development of cases to gastric cancer was statistically significant (P <0.001) among the patient groups. But there was not statistically significant association between other genotype combination and the gastric cancer (P ≥0.113).

The apparent absence of *bab***A2** and *bab***B** alleles in GC patients raise, however, hopes that larger studies may establish the usefulness of these alleles in guiding patient management.

Table 3: Combination of *bab*A2, *bab*B and *ice*A1 genotypes and clinical outcome

(Presence of a gene= positive, absence of a gene= negative, *P*v: p-value)

*bab***A2**	*bab***B**	*ice***A1**	**GC** (n=9)	**NUD** (n=81)	**PUD** (n=19)	**Total** (n=109)	p-value
Positive	Positive	Positive	0	19	6	25	0.113
Positive	Positive	Negative	0	15	3	18	0.121
Positive	Negative	Positive	0	12	2	14	0.136
Positive	Negative	Negative	0	9	4	13	0.196
Negative	Positive	Positive	3	7	1	11	0.216
Negative	Positive	Negative	0	11	2	13	0.157
Negative	Negative	Positive	6	3	1	10	0.001
Negative	Negative	Negative	0	5	0	5	0.253

Discussion

Various studies have observed substantial differences in incidence and/or severity of gastroduodenal pathologies related to H. pylori which may vary according to geographical regions [26]. Although many factors may contribute to these differences, an obvious contributing factor is the different distribution of pathogenic markers in circulating strains [18]. The clinical relevance of the putative virulence-associated genes of H. pylori and geographical region remains controversial. Other factors that influence the risks for atrophy and cancer in the presence of infection may be related to the time when infection occurred, to other environmental factors and to the host genetic variation [27]. In particular single nucleotide polymorphisms in genes that influence bacterial handling via pattern recognition receptors appear to be involved, further strengthening the link between host risk factors, H. pylori

incidence and cancer[28]. In the present study we exploited this situation to study the relationship between selected virulence genes of H. pylori and the clinical status. Our results clearly support the notion that further studies aimed at establishing the negative predictive value of the presence of babA2 and babB alleles for GC development are warranted.

The cagE is a pathogenicity biomarker of H. pylori. A survey of previous studies suggested that the cagE prevalence is different around the world [29]. The general importance of cagE is best illustrated by its high frequency in GC patients as demonstrated by studies performed in patient populations derived from India (100%), Turkey (81.8%) and Thailand (93.8%)[30]. however, the prevalence of cagE gene in this study was only 55.9%, markedly different from the results obtained in the aforementioned countries [31]. In the present study cagE-positive isolates were slightly more detected in isolates from peptic ulcer dyspepsia patients PUD (68.4%) patients, but the potential significance of this finding, if any, remains to be established. The prevalence of cagA gene in our cohort is 70.6%, which resembles the situation in Western countries (Yamaoka et al., 2002), but is markedly lower than that observed in East Asian countries where cagA is present in more than 90% of cases (Uchida et al., 2009). Like cagE, cagA-positive isolates were enriched in samples obtained from peptic ulcer dyspepsia patients, albeit not in a statistically significant manner. Nevertheless, our findings support previous studies (Uchida et al., 2009, Wu et al., 2003) and should prove interesting to include our data in a meta-analysis of virulence genes in this respect. Our results show that the prevalence of iceA1 and iceA2 genes in isolates was 42.2% and 13.2%, respectively. These results are in agreement with previous studies showing that the iceA1 gene is prevalent in Japanese, Korean and The Dutch patients (Ito et al., 2000, Ko et al., 2008, Shiota et al., 2013) conversely the iceA2 allele was predominant in the United States and Colombia. In our cohort, the relation of the presence of iceA1 and the development of cases to gastric cancer was statistically significant (P=0.008). But our data showed that there was no significant association between iceA2 and GC compared with PUD or NUD. However, several studies have reported different results, as the iceA2 gene was detected to be predominant genotype in these studies (Aghdam et al., 2014, Biernat et al., 2014).

The babA is one of the mediators for the attachment to gastric cells by H. pylori[32]. More recent analysis of babA2 as a virulence marker has produced conflicting data on the usefulness of babA2 expression in predicting clinical outcome, which is most

likely dependent on the geographic origin of the H. pylori strains. Survey of previous studies on Portuguese, Thai and India populations showed that, babA2 is not a marker for peptic ulcer disease or gastric cancer [12, 26, 33]. However, several studies have reported different results for strains isolated from Turkey or Germany [26, 34]. Our study suggests that babA2 "although quite prevalent" is associated with reduced propensity to develop GC and is thus associated with less oncogenic H. pylori. Similar results were obtained concerning babB.

Our results showed the relation of babA2, babB negative and iceA1 positive genotype and the development of cases to gastric cancer was statistically significant. Further studies are required to determine the functions of babA2 and babB and their relationship with disease outcome, and whether the presence of these alleles indicates the presence of bacteria unlikely to confer progression towards GC.

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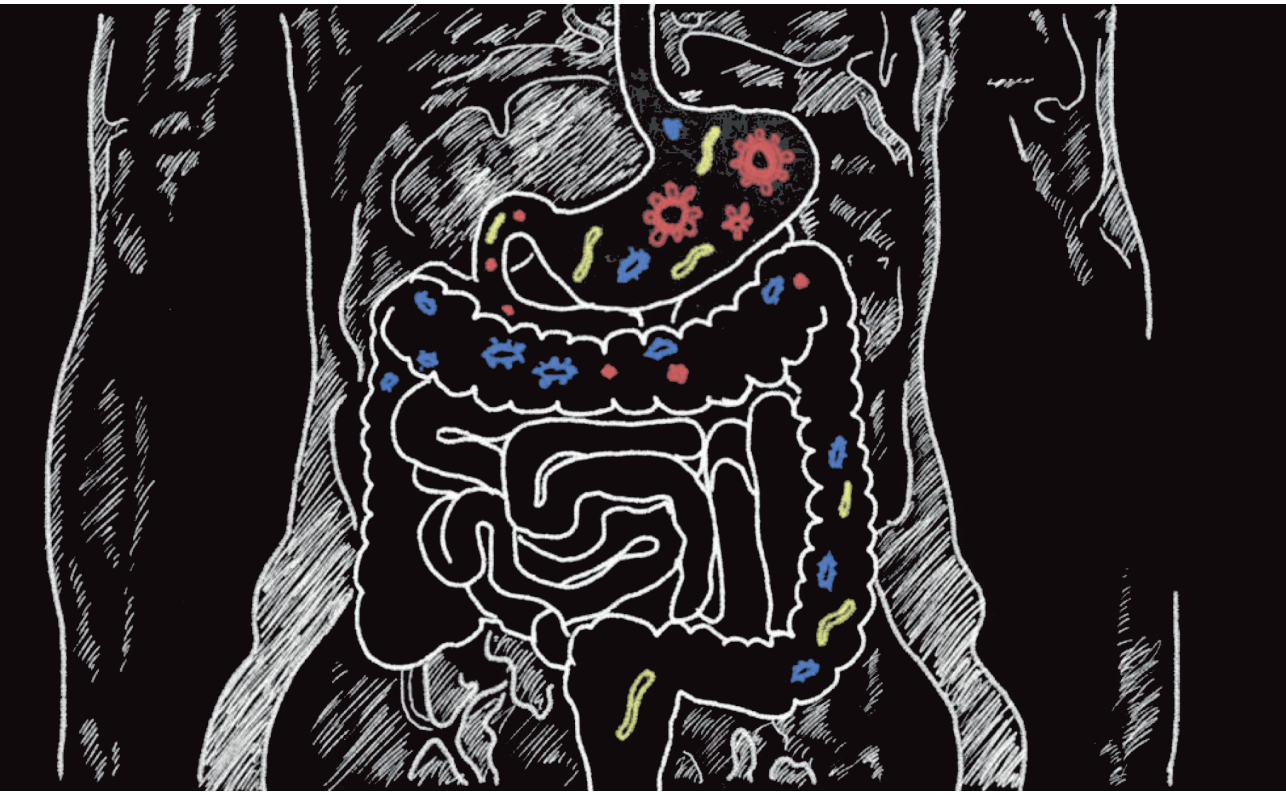

Chapter 7

The Immune System in Space: Health from above?

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Life Sciences in Space Research, in press

Abstract

Among the most quoted reasons for defending spending by governments on space programs is the scientific knowledge and advances we gain that also have a positive impact back on earth, for which altered immunity in microgravity is one of these. Although the mechanisms involved remain primarily obscure and controversial, detection of these mechanisms may well be of importance that supersedes the field of microgravity research itself: autoimmune disease poses an ever more significant challenge to society, and novel therapeutic angles to combat immune system overactivation are urgently called for. Thus it is being claimed that the extensive efforts society makes about supporting space-based experimentation can be justified from the promise that identification of the molecular mechanisms by which microgravity interacts with the immune system will provide new clues as for how to therapeutically interfere with immune overactivity. Although data are limited, ,the effects on signaling most likely involve alterations in Rac signaling and thus microgravity provides new insight into the role of Rac and especially its downstream impact on cytoskeletal architecture in immune signal transduction. This has significant implications: both functional in studies which suggest that at least for one autoimmune disease, in case of Crohn's disease defects in innate immunity are causative, leading to excessive innate responses. Thus indeed it is at least theoretically possible that useful information may be obtained from space experimentation on human immunity.

Keywords: Microgravity; Immunity; Signal transduction; Space travel; Innate immunity; Adaptive Immunity

Introduction

Earth's gravitational field predominate life evolution and has contributed to all biological systems architecture determinedly. It is hence not astonishing that abrupt variations in microgravity can lead to divergences in the regular functions of life and immune system. According to Brian Crucian in the NASA biological studies. The immune system of space crew members can be impaired by things like microgravity, radiation, microbes, and altered sleep cycles. And if conditions persist for a more extended period during a space mission, it could mimic chances of higher risk of infection, autoimmune disease and hypersensitivity for space explorers.

Microgravity influences the human body system actively which might lead to wide range of symptoms that are deconditioning such as immune system deterioration, impaired cardiovascular system performance, demineralization of the bone and neurovestibular altered perception[1]. In short-term, space traveler presented depression of immune system, T lymphocytes reduced activation and virus latent reactivation[2, 3]. Prevailing strategies to establish a framework of infrastructure for profitable spaceports with the goa,l of creating spaceflight accessible and affordable to retail consumers advocate that we can anticipate spaceflight to increasingly become open to the public in nearest future[4]. A vital means to travel across the globe will be through suborbital flights. As increasing commercial enterprises are already booking spaceflight experiences for paying customers, physicians may expect patients are inquiring about the risks or fitness for space travel [4, 5]. There is a need for extensive medical screening of intended travelers since a trip to the International Space Station takes two to three weeks[6]. Furthermore, aside from the physiological[5] and clinical implications of human spaceflight[4], exposure to microgravity[7], as well as to the hastening forces during launch and landing[8], all this will have an impact on both human microbiomes and immune system. In addition to microgravity, humans encounter cosmic and solar radiation which is a unique environmental condition during spaceflight. Astronauts experience an increased risk of infection due to the impaired immune system as a result of changes in space ecological condition.

The constant pathogenic organisms threat and the resulting evolutionary pressure to develop defenses against these challenges have resulted in an array of collaborative defensive cell systems, consisting of amongst others phagocytes and versatile B and T cell frameworks which are collectively summarized under the wide denominator immunity. The human immune system, similar to all cellular frameworks in our body,

developed in the consistent nearness of the earth' gravitational field. Thus it is not inconceivable that acute absence of this external force, as occurs during space flight, would influence functionality in this system. Diminished activity of the immune system is a well-documented effect of microgravity on the human body, for which precise mechanisms have yet to be determined. Importantly, although this diminished activity of the immune system is likely to become a problem concerning the extended missions presently being premeditated and planned (particularly an operated mission to Mars may yield specific challenges in this respect), space-related immunosuppression is well-endured. Over forty years of knowledge with manned space flight have now overwhelmingly established that man can effortlessly stay alive and work in circumstances that are weightless, notwithstanding the continued repression of the body's immune functions. This suggests that simulating immunosuppression by space-related circumstances on earth may be a reasonably straightforward approach to immune system suppression as would be beneficial in patients suffering from excessive immune activation, as is the case with an autoimmune disease. Some studies also confirmed that space-related immunosuppression even exists in simulated models of microgravity (sμg)[9] demonstrating immunosuppression in splenocytes. This illustrates the usefulness of sμg models in novel illuminating mechanisms that control T-cell function and in enhancing spaceflight research in general. Eventually, creating awareness into monitoring lymphocyte pathways gained from sμg and spaceflight research will profit not only future astronauts but also those on earth who suffer from immune maladies[10].

This promise of microgravity has driven us in the past years to heavily invest in developing experimentation suited for space research, employing immune cells isolated from human volunteer peripheral blood, and three times the team has delivered experiments to a space-going vehicle. The current review is directed in maximizing the scientific returns of this unique situation by providing the necessary scientific infrastructure about performing experiments and analyzing (employing advanced single-cell technology to extract as much information as possible) the wealth of data expected, delivering a relatively detailed picture of microgravity effects on signal transduction in human immunity.

Historical background of the concept that microgravity influences human host defense.

Probably ever since the early days of a space probe, it has been suggested that extra-atmospheric humans suffer from the reduced immune function. Although, as a consequence of the secrecy concerning this topic during the Cold War era actual medical data and scientific reporting on the early space exploration is difficult or even impossible to obtain (especially from the ex-Soviet Union). Some scarce scientific reports and informal communication do suggest, with regard to the Russian experience, immune dysfunction was a concern [11] and in the American experience it certainly was: the Mercury program-flown chimpanzee Enos died in 1962 from Shigella infection, quite soon after his space trip on November 29, 1961 (although now many investigators doubt whether his death was related to his space travel). Likewise, the death of the pig-tailed monkey Bonnie on biosatellite 3 (the first primate satellite) raised, at the time, wide-spread concern concerning the immunological safety of space travel. Although now many feel that the death of Bonnie is to be attributed to "over-instrumentation" of the unfortunate animal[12]. Minor bacterial and viral infections were not uncommon. Among the Apollo astronauts, a range of diseases was reported, mainly of the eyes, skin and respiratory tract (including stomatitis, pharyngitis, recurrent inguinal and axillary infections). The astronaut Fred Haise developed a Pseudomonas aeruginosa urinary tract infection on Apollo, and this case was prominently suggested at that time to be a space-travel specific increased susceptibility towards viral infection via a publication in the authoritative medical journal JAMA[13]. Thus at that time, space-travel was associated with the diminished functioning of the immune system. However, the effect of space travel is probably not blank immunosuppression, and when appropriate measures are taken such as vigilant hygiene (which is relatively easy in the confined and isolated setting of space going vehicle) and a proper pre-flight medical examination of the crew, acute immunosuppression-related medical problems remain quite rare. Nevertheless, overt signs of mild immune suppressions are evident, for example, the re-activation of the varicella-zoster virus[14], herpes virus[15], and Epstein-Barr virus[16], but remain subclinical. It cannot be excluded, however, that during a long-term mission, like the proposed manned flight to Mars, diminished immunity could become a problem. And thus in the context of such long-duration space flight, knowledge on the molecular mechanisms that are responsible for space travel-associated immunosuppression have a practical importance that exceeds the scientifically fundamental question as to the interaction of the gravitational earth field and the human immune system.

Purpose and structure of this review

Spaceflight causes deregulation of the human immune system and may compromise a crews wellbeing as during missions their health is continuously challenged by environmental conditions. Consequently, it is crucial to comprehend the biology of immune modulation under spaceflight circumstances possibly providing us with a means to uphold immune homeostasis under such conditions[17]. Life in the sterile and confined surroundings of a spacecraft in the presence of microgravity and cosmic radiation may alter the equilibrium between the human body and the microbiome. The combination of a deregulated immune system and an altered microbiome increase the risk of microbial infection and potentially the development of conditions related to reduced tolerance. This risk is further heightened by an increase in virulence of pathogens in microgravity. Health status of astronauts might potentially benefit from maintaining a healthy microbiome by specifically managing their diet on space in addition to probiotic therapies. This review focuses on the current knowledge/understanding of how spaceflight affects human immunity and microbiome[18]. Some of the impact on immune function certainly relate to psychological factors (cosmonauts can be expected to suffer from stress, wearing working schedules and anxiety), disturbance of circadian rhythms, environmental exposure to cosmic radiation and magnetic fields and substantial ergonomic discomfort (cramped conditions, inadequate lifestyle and nutritional regimen etc.)[15, 19]. However, there are good reasons to assume that the weightlessness itself negatively influences immune competence of the human body. Experimentation with crew members of the Apollo and Soyuz missions[20] showed reduced reactivity of peripheral blood lymphoid cells. And a landmark experiment conducted in Spacelab 1 in 1983 demonstrated a near absent activation of peripheral blood leukocytes upon exposure to concanavalin A (Con-A) when compared to identical ground controls[21]. This observation sparked wide-spread interest into microgravity effects on the immune system. Although this original view has been confirmed in elegantly designed experiments in which controls were incubated in an in-flight 1×g reference centrifuge[22], many of the details as to how gravity and immune cell function interact remain obscure.

In this review we shall look at the different levels at which microgravity might interact with the human immune system and analysing the available evidence, we shall conclude that especially T cell[15, 21-24] and Natural Killer cell responses are negatively influenced[14-16, 19], but that innate immunity and especially granulocyte

responses are upregulated[25, 26]. Next, we would review the causes of these phenomena and conclude that they probably do not derive from differences in the extracellular milieu[27, 28] (which would have been a possibility in view of the significant effects on the bone marrow niche due to loss of bone[29-31]), but are the result of changed intracellular signal transduction under microgravity[32, 33]. Although data are limited, the effects on signaling most like involve alterations in Rac signaling[34] and thus microgravity provides new insight into the role of Rac and especially its downstream impact on cytoskeletal architecture in immune signal transduction[29]. Finally, we shall discuss the implications for human disease. These consequences are quite impressive: both functional studies[35] and GWAS[36] suggest that at least for one autoimmune disease, in case of Crohn's disease defects in innate immunity are causative, leading to excessive innate responses. Thus indeed it is at least theoretically possible that useful information may be obtained from space experimentation on human immunity

Microbiomes and humans

The human body is the living space of microbial symbionts which is ten times more in number than all Homo sapiens cells[37]. The expression "microbiota" alludes to the organisms that live in a particular section (e.g., the human body, the gut, the dirt, and so forth.). The cumulative genomes of the total microbiota in the human body are alluded to as the "human microbiome"[37, 38]. The microbiome impacts biochemical, physiological and immunological pathways and is the principal line of protection from various diseases. The cooperation between the gastrointestinal frontier and the microbiome give off an impression of being a complex system that helps with keeping up appropriate immune capacity in the gut[39, 40]. The intestinal microbiome shapes the gastrointestinal insusceptible framework[41, 42]. Stable and pathogenic microorganisms go after supremacy[43, 44]. A balance between useful and potentially harmful microscopic organisms is considered normal and adds to a robust and healthy human gut[44]. There is growing validation of the relationship between the changes in the balance of gut flora and several diseases[45].

Microbiomes and spaceflight

The last decades have seen growing concerns about the effects of microgravity on the microbiome of astronauts during long-duration stay in space. Various studies[51], have reported changes in gut microbiome physiology and composition. As mentioned the

presence of a healthy gut microbiome is necessary to maintain immune homeostasis. As such the dysbiosis that results from prolonged stay in space may affect the health of astronauts causing higher susceptibility to opportunistic infections, reactivation of latent infections, induction of IBS and increased potential to develop diabetes or even cancer.

A possible explanation as to why this dysbiosis occurs in space is the lack of microbial challenge while staying on board a space craft. Due to the confinement of the living space, a strict hygiene regime is maintained. This does not only concern the living quarters but also the food astronauts eat and the air they breathe. Not risking the chance to get sick by microbial infection, limits the natural competition commensal microbes would normally experience by intake of pathogenic microbes. Consequential some may experience a potential growth advantage altering the balance of the gut flora.

The deleterious effect of prolonged stay in space on the microbiome may be countered by measures such as diet based therapies. These diets include vegetables, fresh fruits and fiber rich diets which enhanced the growth of butyrate-producing bacteria potentially reversing the profile of metabolites associated with human disease. This hypothesis is currently being tested by NASA using a combination of metabolomics, metatranscriptomics, 16s taxonomic profiling and immunological approaches. Alternatively the gut flora may be supplemented by probiotics or possibly fecal transplant pills. However, effects of such strategies still require investigation. Spaceflight long journey to the Mars by astronauts present a lot of challenges of which one among them is their inability to maintain a healthy microbiome population. This microbiome that forms the normal biota of the human intestine helps in protecting the host from infectious diseases, enhance immunity and breakdown of food; A wide range of condition may arise due to imbalance in microbiome compositions these include syndrome of irritable bowel[46, 47], different cancer type[47] and depression[48]. During spaceflight travel, there is a paradigm shift from healthy to disease state which translates to decrease in microbiome diversity and enhance inflammation. cosmic radiation and Stress are among typical conditions experience during space flight travels[49, 50]. Changed in physiology of bacteria and promotion of dysbiosis of microbiome status[49].

A successful return mission to the planet Mars takes more than a thousand days considering the most optimal conditions, a duration for which a healthy microbiome is imperative. It is therefore that space agencies have prioritized investigations on maintaining a healthy and balanced microbiome. Questions that need answering are:

Firstly, how to balance natural microbiota diversity during long term stay in space. Secondly, how can we combine a low pathogenic environment for the crew of a space craft without altering the normal composition of the commensal flora. Third, how can we restore normal gut flora after partial eradication by for example antibiotic treatment. And lastly, to what extend does cosmic radiation damage the diversity of the microbiome.[51].

Altered cytokine production does not provide a mechanical explanation for microgravity-induced immune suppression.

Clinical strategies to interfere with the human immune system can involve either change in the extracellular or modification of cellular biochemistry operative in the immune effector cell. Examples of the former like the modification of cellular biochemistry by increasing the blood concentration of immunosuppressive corticoids[52] or the bio-neutralization of pro-inflammatory cytokine Tumour Necrosis Factor[53] by intravenous administration of anti-TNF antibodies are used with great therapeutic success in the treatment of autoimmune disease. An example of the latter is inhibition of Ca2+ transient-dependent activation of immune effector cells by cyclosporin-like compounds in transplantation medicine [54]. The effects of microgravity on immunity could be derived alternatively from altered cytokine production, thus altering the extracellular environment in which an immune response has to develop, or from autonomous cell mechanisms, in which the cellular response to extracellular cues is changed. The experiments with stimulation of ex vivo T cells would point to the latter; such reactions do in general not prominently involve alterations in cytokine milieu. Indeed, work in bone suggests that levels of immunosuppressive cytokines like TGF-ß are decreased instead of increased during microgravity and thus unlikely to account for space-related immunosuppression[30]. In agreement with the notion apparently that the effects of microgravity are cell-intrinsic rather than the consequence of an altered cytokine milieu, is the observation that in primary isolated rat lymphocytes, leptin (a hormone that famously regulates appetite[55], but is also prominent regulator of immunological[56] and histo-static[57] functions in the endodermal mucosal compartments) loses its capacity to stimulate proliferation under simulated microgravity[27]. Also efforts to measure the concentrations of cytokines and other immunologically relevant hormones in the peripheral blood of astronauts or experimental space-going rodents have never yielded convincing reductions in the profile of these humoral factors (e.g. Miller et al. J. Appl. Physiol. 78: 810-813), although also during investigations under conventional 1xg

conditions it is sometimes tricky to detect reflections of pathology in the cytokine profile of peripheral blood in the absence of fulminant disease[58]. Nevertheless, despite 30 years of frantic research and experimentation, there is no evidence that microgravity-related immunosuppression can be linked to changes in the external milieu.

It is important to note however, that changes in intracellular signalling and extracellular milieu can never be regarded separately, as diminished intracellular signalling will result in diminished cytokine production, in turn affecting downstream responses and feedback loops, e.g., Con-A stimulated interleukin 2 secretions by T lymphocytes is negatively affected by simulated microgravity and this will without doubt aid the reduced mitogenic responsiveness to Con-A during microgravity in this cellular compartment[59]. Nevertheless, altered responsiveness to extracellular cues rather than a difference in the extracellular environment explains why immune cells behave differently during autoimmunity, focussing studies towards the influence of microgravity on cell-autonomous mechanisms. Some studies also found that LPS-induced TNF-α expression in mouse macrophages was significantly suppressed under simulated microgravity. NF-κB activation and TNF-α mRNA stability were resistant to microgravity. The repressor of TNF-α promoter, HSF1, which was activated under simulated microgravity, may be one of the critical mechanisms for the reduced TNF-α expression in macrophages under microgravity[60]. The oxidative burst reaction in mammalian cells, necessary for example for the internalization and degradation of bacteria, depends on the presence of gravity[61]. The release of reactive oxygen species (ROS) during the oxidative burst reaction depends on gravity conditions. ROS release is 1.) reduced in microgravity, 2.) enhanced in hypergravity and 3.) responds rapidly and reversible to altered gravity within seconds. We substantiated the effect of altered gravity on oxidative burst reaction in two independent experimental systems, parabolic flights, and 2D clinostat/centrifuge experiments. Furthermore, the results obtained in simulated microgravity (2D clino-rotation experiments) were validated by experiments in real microgravity, in both cases showing a pronounced reduction in ROS. Our analyses indicate that gravity-sensitive steps are located both in the initial activation pathways and in the final oxidative burst reaction itself, which could be explained by the role of cytoskeletal dynamics in the assembly and functionality of the NADPH oxidase complex[61].

Space travel and microgravity effects on immune cell function are highly cell-type specific.

Evidence that the immunosuppressive effects of space travel in general and microgravity, in particular, can be attributed to specific cell-autonomous mechanisms which impact on different compartments of the immune system, has over the past three decades slowly become more-and-more evident. Advancement in this field has been slow due to the apparent lack of experimental opportunities and the technical difficulties in the analysis of scientific work on the immune system in microgravity. The notion, however, that space travel in general and microgravity, in particular, are immunosuppressive was much fostered by the observation that Con-A stimulation of T cell compartment resulted in reduced mitogenic responses in this cell type under microgravity. Thus a lot of attention has been focused on this branch of immunity. Typically studies show that cell division is reduced between 50–89% at 0×g when compared to 1×g controls either in space or during simulated microgravity[22].A variety of studies indicates that microgravity interferes with the functionality of the NK cell compartment. NK cells isolated from astronauts are deficient in target cell killing in ex vivo assays (hence out of the context of a possible immunosuppressive cytokine milieu, suggesting that cellular factors are essential here)[15]. Although not well studied, maturation of dendritic cells, significant for the instruction of T cells in adaptive immunity is somewhat impaired under gravity[62]. In contrast neutrophil action seems to be enhanced under microgravity. Cosmonauts spending 1 to 22 months in microgravity (occupants of the Russian Mir space station) display increased granulocyte superoxide production and enhanced levels of surrogate markers for such increased granulocyte activity like elevated erythrocyte superoxide dismutase activity and glutathione oxidation[25]. Hence, if anything, immunity in the neutrophil compartment is slightly stimulated rather than inhibited by microgravity. The notion that space travel can specifically interfere with individual branches of protection without negatively influencing granulocyte immunology adds to the interest that identifying the molecular targets of microgravity may have. Evidence that defects in innate immunity contribute to the pathogenesis of the autoimmune disease is rapidly accumulating[63]. Recent genome-wide associations studies (GWAS) have been instrumental in identifying novel genetic risk factors predisposing to Crohn’s disease and many of the alleles involved confer reduced activity in the innate immune system [36]. In line with these genetic studies, patients with autoimmune disease were shown to exhibit diminished innate immunity as compared to healthy individuals[35] and genetic deficiencies in neutrophil function are highly similar to and clinically even

often mistaken for autoimmune disease[64]. Thus, for the treatment of autoimmune disease, immunosuppression in lymphocyte compartment without concomitant innate immunosuppression appears much more attractive as the blank immunosuppression, such as occurs with, for instance, steroid treatment, the mainstay of current treatment of autoimmune disease. Apparently, as microgravity does not seem to negatively affect neutrophil-dependent immunity, identifying the targets of microgravity on human immunity may prove exceedingly useful for identifying such targets.

Altered signal transduction is important for microgravity effects on immune function

Thus the available evidence suggests that different cell types alter their characteristics during microgravity and that these effects cell-autonomous, i.e., they do not majorly involve differences in the extracellular milieu, but involve an altered intracellular reaction to an extracellular stimulus. The most straightforward fashion in which such an effect could occur is that the interaction between extracellular cues and their corresponding receptors is disturbed during microgravity. This, however, does not seem to be the case, for instance, the binding of Con-A to T cell membrane glycoproteins remains unchanged, and patching and capping are only minimally retarded at 0×g[65]. Although not involving immunologically relevant cells, also the observation that the otherwise microgravity-sensitive EGF response in A431 cells does not require altered EGF receptor clustering on the surface of A431 cells supports this notion[66]. Thus it seems that subsequent intracellular biochemistry is the target here. Support for this idea comes from the observation that induction of early genes (i.e. genes which are activated transiently and rapidly in response to a wide variety of cellular stimuli and seem to serve an alarm bell for the nuclear machinery that alteration in its transcriptional programming are imminent and form the first nuclear reaction to an extracellular stimulus)[34, 32, 33], apparently limiting the possible interaction points of microgravity with immunity with the direct signaling machinery associated with transmitting the extracellular signals to the nucleus and possibly not necessarily involving altered reactions to secondary nucleus-derived signals.

Altered signal transduction in immunological cells may be dependent on diminished Rac activation

The nature of the effects of microgravity on cellular signal transduction remains unclear. It is tempting to speculate that these results may involve alterations in signaling

through the small GTPase Rac. Rac is a pleiotropic regulator of many cellular processes, including the control of cell growth, cytoskeletal reorganization, and the activation of protein kinases and phospholipase A2[67]. Gene expression profiling in osteoblasts identified especially cox-2, c-myc, bcl2, TGF beta1, bFGF and PCNA as being negatively targeted by microgravity[34], which are genes whose expression is downstream of the Rac effector pathways involving activation of the p38 MAPK and Jun-N-terminal kinases. Production of phospholipase A2-dependent pro-inflammatory lipid mediators is negatively affected by microgravity (Gravit Physiol. 2004 Jul;11(2): P41-2. Effect of simulated microgravity on PGE2-induced edema and hyperalgesia in rat paws: pharmacological data and biochemical correlates. Peana AT, Bennardini F, Buttu L, Pippia P, Meloni MA, Stuffler RG, Maccarrone M.). Also, the cytoskeletal effects of microgravity on growth factor-induced cytoskeletal remodeling seem consistent with an impact on Rac activation[68]. (Describe effects of expression of Rac. Inhibitory action of microgravity would fit well with differential effects of microgravity on alternative immunological compartments, whereas moderate Rac inhibition leads to diminished action of the adaptive immune system, such inhibition enhances effector function in phagocytes). Hence microgravity mirrors essential aspects of the known consequences of Rac inhibition on the immune system and it should prove interesting to directly assess the influence of space flight on GTP-loading of this GTPase or the phosphorylation state of its direct effectors, the p21-activated kinases (or PAKs).

Effect of space flight-associated microgravity on stress-activated protein kinases in innate immunity.

Spaceflight actively moderates human immunity but is in general well tolerated. Elucidation of the mechanisms by which zero gravity interacts with human immunity may provide clues for developing rational avenues to deal with exaggerated immune responses, e.g., as in autoimmune disease[69]. The human immune system, like all cell biological systems in our body, has developed in the continuous presence of the earth's gravitational field. Thus it is conceivable that acute absence of this external force, as occurs during space flight, would influence functionality in this system. Indeed since the early days of space exploration, it has been evident that extra-atmospheric primates and humans experience reduced immune function[13, 21, 70].

Activation of p38 map kinase is not affected by space flight associated microgravity.

One of the most notable biochemical changes following LPS challenge is the phosphorylation and thereby activation of p38 MAP kinase[71, 72]. Inhibition of p38 MAP kinase protects against endotoxemia in LPS challenged healthy volunteers[72], although clinical implementation of pharmacological p38 MAP kinase inhibition has not proven viable because of side effects and extreme vulnerability towards bacterial infection. Various genes, like PRKCA22, whose expression is known to be exquisitely sensitive to microgravity are identified to be regulated by p38 MAP kinase[73]. Hence, it is conceivable that activation of p38 MAPK is impaired during microgravity. As expected, the onboard 1xg control displayed p38 MAP kinase activation. In contrast, activation of p38 MAP kinase was not impaired in microgravity-exposed monocytes, if anything p38 MAP kinase activation appears to be increased. Thereby suggesting that space flight-associated microgravity does not interfere with LPS-induced pro-inflammatory signal transduction per se and its effects, if present, are restricted to specific signaling pathways.

LPS does not activate Jun-n-terminal kinase during space flight associated microgravity.

In general inflammatory stimuli coactivate p38 MAP kinase and Jun-N-terminal kinase, as activation of both kinases displays similar kinetics[74]. Nevertheless, they are activated by different upstream kinases (MKK3/6 and MKK4/7 for p38 MAP kinase and Jun-N-terminal kinase, respectively[75]. Thus, at least theoretically, the possibility exists that space flight-associated microgravity targets Jun-N-terminal kinase without a concomitant effect on p38 MAP kinase. Surprisingly, during microgravity, the capacity of LPS to stimulate Jun-N-terminal kinase activation is lost, whereas monocytes stimulated with LPS during the onboard control condition were utterly Jun-N-terminal kinase activation proficient. Thus, microgravity evokes a dichotomy in immunological signaling, allowing p38 MAP kinase activation to proceed, but incompatible with LPS dependent Jun-N-terminal kinase activation. Although it does not involve immunologically relevant cells, the observation that the induction of c-Jun in A431 cells by epidermal growth factor is absent in microgravity[32], fits well with our findings. Especially since it has been well established that Jun-N-terminal kinase is involved in the induction of c-Jun and p38 MAPK is not.

Effects of space flight-associated microgravity on erk activation.

Although less pronounced than the effect on Jun-N-terminal kinase, inhibitory influence of microgravity on the capacity of LPS to provoke activation of the extracellular regulated kinase (ERK) was observed[69]. This corresponds with recent findings in T cells that show a minor effect of microgravity on ERK phosphorylation in unstimulated T cells[76]. Thus, the impact of microgravity is not solely restricted to the Jun-N-terminal kinase. Diminished activity of the immune system is among the foremost effects of space flight on the human body[77]. Importantly, this reduced activity of the immune system is well-tolerated. Over forty years of experience with manned space flight have now conclusively demonstrated that man can easily survive and work in weightless conditions, despite the chronic repression of the body's immune functions[78].

This suggests that mimicking space-related immunosuppression on earth may be a fairly safe way of suppressing the action of the immune system. Although experimental requirements allowed for only 6 minutes of microgravity, there is good evidence that even a relatively short inhibition of kinases can have a substantial effect on the functionality of the innate immune system[79]. Therefore we feel that the observed effect on Jun-N-terminal kinase is relevant to the explanation of space flight-associated immunosuppression. The marked dichotomy between p38 MAP kinase and Jun-N-terminal kinase is consistent with these observations: p38 MAP kinase is essential for fighting immediate bacterial threats, and thus its inhibition causes undesirable side effects in these and other respects[80]. On the other hand, the Jun-N-terminal kinase is more associated with chronic inflammatory responses[81, 82] whose inhibition represents a clear and for now unresolved clinical need.

Conclusion and future direction.

Immune system of space travellers diminishes upon spaceflight. Importantly, although this reduced activity of the immune system is likely to become a problem concerning the more extended missions currently being planned (especially a manned mission to Mars may yield specific challenges in this regard), space-related immunosuppression is

well-endured. Over forty years of experience with manned space flight have now conclusively demonstrated that man can easily survive and work in weightless conditions, despite the chronic repression of the body's immune functions. This suggests that mimicking space-related immunosuppression on earth may be a relatively suppressing the immune system in patients suffering from excessive immune activation, like autoimmune disease, hence identification of the machinery responsible could provide significant benefit for earthly health care.

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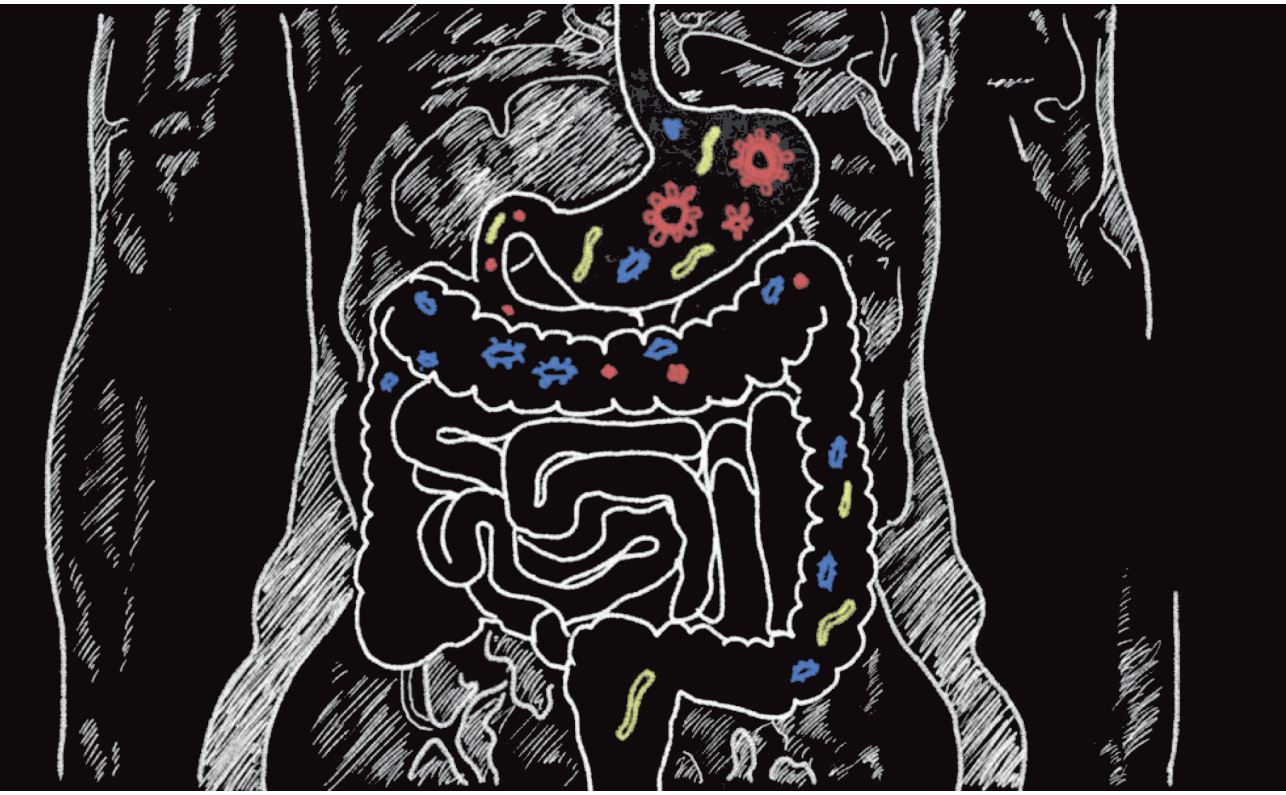


Chapter 8

Summary, General Discussion, and Future perspective

Summary

Cancer is the generic term used to describe a group of diseases that involves a pathology characterized by abnormal and uncontrolled cell proliferation. Mechanistically, this group of diseases find its etiology in a disturbance of the cellular signaling pathways that in physiology tightly control tissue compartment size homeostasis but in cancer lose feedback control. The resulting growth physically impairs body function and can provoke substantial pain and suffering. To cure disease or at least improve quality of life, it is important to know how cancer patients respond to different treatment options and to develop new treatment modalities as current ones are often still largely inadequate.

Theoretically, one might assume that the changes in genetic make-up that are associated with the cancerous process would create neoantigens and thus tumor cells should be subject to attack by the body's immune system. Although, there is evidence that many tumors are to a certain extent constrained in their growth by immunity, evidently this control fails in many patients. In this context it is of substantial significance to study the tumor microenvironment as a whole, because it has valuable prognostic potential with respect as to how the disease course will react to the immunogenic mutation load.

The studies presented in this thesis on gastric cancer appear to at least partially support this concept. The survival rate of a gastric cancer patient is dependent on the associated immune parameters within the tumor microenvironment. I feel it constitutes a prognostic marker that can be used to evaluate if an individual is at risk of cancer development, and if disease has developed can assist in treatment selection. An important goal in this respect, that has not yet been satisfactorily reached, is to determine a mechanistic signature (expression patterns of immunomodulatory molecules, ratios of immune suppressive cell types over immune effector cell types etcetera) that is associated with either success or failure in immunotherapy. The absence of such a predictive signature now greatly hampers implementation of immunocheckpoint therapy in gastric cancer, hence patients that might benefit from such therapy are being denied access to it whereas patients not benefiting from immunocheckpoint-directed therapy suffer its side effects. Nevertheless, the emerging capacity to better characterize both cancer cells for neoantigen expression and to precisely phenotype the tumor micro-environment will certainly improve matters in this respect. It is important to stress, however, that final

success in the battle against gastric cancer will depend not only on improved therapy in these late phases of disease. Gastric cancer remains an important contributor to overall mortality and especially cancer-related mortality when viewed globally. To deal with this problem it will be necessary to improve prevention, e.g. by widening opportunities for more active screening of subjects which are predisposed to develop gastric cancer, to improve the efficacy of screening procedures involved and to better understand the gastric cancer process as to open novel avenues for the rational treatment of disease, in particular opportunities for preventive therapy when atrophic gastritis develops should exist. My thesis has tried to make contributions here. My findings will be summarized and discussed in this chapter. I shall also provide future perspectives and issues that need to be addressed in additional studies.

In chapter 2 I have prepared a comprehensive review of the relation between hedgehog signaling and (development of) gastric cancer. Some of the related aspects I also reflect on in chapter 1, Hedgehog signaling has a pivotal role in the homeostasis of the healthy stomach, whereas loss of Hedgehog signaling is an important event in neoplastic transformation of the stomach. Loss of Hedgehog production in the parietal cells during atrophic gastritis drives excessive production of gastrin (among other events) in turn resulting in excess gastrin production which is an important disease-promoting occurrence. Once cancer has been established Hedgehog signaling may support the cancer process and especially have a trophic influence on the cancer stem cell compartment. Thus modulation of Hedgehog signaling with respect to gastrointestinal cancer development is pivotal at different phases of the gastric cancer process. Although Hedgehog antagonists have been used in different cancer types, the usage of Hedgehog antagonists for gastric cancer in clinical trials is still in its infancy. New therapeutic strategies need to be explored. Such development is hampered by the lack of knowledge on the mechanistic details of Hedgehog signaling and this aspect of carcinogenesis in the stomach and elsewhere in the gastrointestinal tract will be dealt with elsewhere in this thesis.

In chapter 3 I further evaluated immunotherapy checkpoint inhibition in gastric cancer. Gastric cancer stands out amongst other cancers as particularly aggressive and as a disease in which chemotherapy alone cannot satisfactorily help to change the final outcome due to constrained efficacy. A critical factor that guides both tumorigenicity of precancerous lesions and the outcome of cancer itself, is immune signaling and how it relates to heterogeneity of gastric cancer (thus not all cells may express the same neoantigen repertoire). We still cannot, however, distinguish those

tumors that can potentially be targeted by stimulation of immune monitoring. This will require full supplementation of information which is still lacking with respect to the multifaceted association between cancer, the tumor surroundings, and the immune system.

Anti-PD-1, Anti-PD-L1 and Anti-CTLA-4-therapy targeting immune checkpoints have delivered hopeful outcomes in some preliminary clinical trials. However, we need to await the results from various ongoing and future clinical trials to properly address its full potential in gastric cancer. Also the possibilities of several synergistic methodologies that may have a beneficial effect (vaccination, cytokine infusions etcetera), either with immunotherapy alone or in further combination with chemotherapy need to be addressed and indeed it appears that such investigations are now starting. Furthermore, plans to ascertain the possibilities offered by potential prognostic biomarkers with respect to the outcome of immunocheckpoint therapy in gastric cancer have already been launched, and I eagerly await the future results of such efforts. In this respect my conclusion is that understanding immune response patterns and better control of immune-therapy-associated side effects will prove vital for widespread adoption in clinical practice for the drugs whose use is now mostly restricted to clinical trials. From the currently available data and its evolving momentum a promising efficacy of immunotherapy agents is revealed but I hope that ongoing clinical trials and preclinical efforts will address the unanswered question I listed above.

In chapter 4 I further explore the mechanistic details that drive Hedgehog signaling, which is an essential morphogen in explaining both physiology and pathology in the gastrointestinal tract. An aspect that has received relatively little attention in the main text of this chapter is that my findings describe a novel signaling cascade functioning through the SRC Homology 3 Domain-containing proteins which, I feel, may be activated directly by Hedgehog ligands but further investigation is necessary to substantiate this notion (e.g. through better analysis of the effects of Hedgehog on the kinome of Patched-deficient fibroblasts, which was not rigoursly done in this thesis). Together, I suggest that Sonic Hedgehog can stimulate Src activity via types I and type II noncanonical signaling pathways and that the latter may resemble ther effects of Hedgehog in axonal guidance in development. Thus I propose a model on the role of Smo interaction with Rho signaling that not only has relevance for the model systems described in this chapter but may also be relevant for formation and regulation of dendritic projections in the neural system (or, who knows, even in

projections of dendritic cells in immunity). Upon activation of the Src-Rho-Rac cascade by Hedgehog, actin cytoskeleton reorganization takes place independent of the activity of any transcription factor and necessary for the functionality of Sonic Hedgehog in physiology. This provoking idea suggests that Hedgehog signaling might regulate a cohort of physiological processes involving fluctuations of Ca2+, acutely affecting membrane potential and Ca2+-dependent signaling pathways, as my promotor showed during his PhD research on the link between actin reorganization and calcium fluxes. But obviously further research is necessary to substantiate this notion. My findings on the new role of Hedgehog in actin cytoskeleton reorganization has thus prompted me to suggest further studies on the interactive parts of Sonic Hedgehog with other morphogenetic cascades during development and formation of dendritic projections.

In chapter 5 gastric atrophy has been described as a precancerous condition and can be evaluated through routine endoscopy. Several studies assessed the agreement level in a variety of methods between endoscopy and histologic atrophy but differ in sensitivity, specificity, precision, and accuracy due to the lack of a gold standard to compare if the grouping is suitable or not. Information from our study on endoscopic grading evaluation has shown a reproducible kappa value, and such grading can be used to predict the precancerous condition of histologic atrophy during routine H. pylori screening practice in Africa and Middle-east.

In chapter 6 we looked at the diversity of Helicobacter pylori genotypes and development of gastric cancer. Our results showed the relationship of babA2, babB negative and iceA1 positive genotype and the development of cases of gastric cancer was statistically significant. Further studies are required to determine the functions of babA2 and babB and their relationship with disease outcome, and whether the presence of these alleles indicates the presence of bacteria unlikely to confer progression towards gastric carcinoma.

As evident from the above, it may prove necessary to stimulate immunity during full-blown gastric cancer. Immunocheckpoint-directed therapy may prove a way forward here, but may not be sufficient to reach the desired level of anticancer immunity. Knowledge of pathways that constrain immunity might help in this respect as the inhibition of those pathways may further stimulate anti-cancer immunity. **In chapter 7** I explore this aspect by evaluating the effect of microgravity as an approach. Space travel is associated with diminished immunity

and thus knowledge of the pathways involved would provide novel targets to stimulate immunity. Excitingly from this analysis Rac/Rho signaling emerges as the pathway targeted by microgravity to inhibit immunity in space travel, implying that stimulation of this pathway might be beneficial in gastric cancer. The same pathway I identified as being downstream of Smoothened in Hedgehog signaling in **chapter 4**. Thus the analysis in **chapter 7** provides further rational for exploring the potential of targeting Hedgehog signaling in gastric cancer. A possibility might be the use of specific Smoothened agonists in combination with Patched antagonists, but requires further exploration.

General discussion

The importance of Hedgehog signaling in many forms of cancer in conjunction with its link to gastric developmental processes has prompted a substantial research effort investigating the potential usefulness of targeting Hedgehog signaling in gastric oncogenesis. As a result, in gastrin-mediated compartment expansion and viral or Helicobacter-dependent gastric carcinogenesis, the role of Hedgehog signaling is now relatively well understood. Furthermore, when the malignant disease is established, the evidence indicates that hedgehog signaling may provoke drug resistance. Now that the Hedgehog inhibitor Vismodegib has come available, which has proved useful in other kinds of cancer (especially basal cell carcinoma), it is tempting to propose clinical trials using this compound for patients who have gastric cancer, and accordingly various of such studies have now been initiated.

Strikingly, however, reports of activating mutations in Hedgehog signaling cascades in gastric cancer are scarce, suggesting that such mutations confer little relative advantage to cancer cells bearing such mutations over those that do not have such mutations. Furthermore, on theoretical grounds, one can expect Hedgehog inhibition to interfere with the clinical effect of conventional medication used to treat gastric cancer.

The data I obtained on Hedgehog signaling may be linked to those I obtained on immuno-checkpoint-directed therapy. Pembrolizumab was shown to have anti-tumor activity in patients diagnosed with advanced, PD-L1 expressing, gastric cancer while causing only limited adverse effects. Suggestive data showed an association between the related immune signature expression and efficacy of the immuno-checkpoint-directed therapy, suggesting this may be a suitable prognostic marker for

identification of patients that will benefit from such a therapy. In addition, characterization of the tumor microenvironment and T cell cytotoxic functions may prove sensitive prognostic markers. Mono-therapy with Avelumab revealed an acceptable antitumor broad spectrum of activity, safety, and efficacy in gastric cancer patients. In a dose-escalation study involving MEDI4736. It has demonstrated an enhance antitumor activity and safety. Ongoing multicenter trials and development of MEDI4736 as a single therapy is ongoing. MPDL3280A was well accepted with enhanced efficacy, safety, and antitumor activity. No adverse events were recorded in the MPDL3280A trial as the interaction between PD-1 and PD-L2 is not affected in response to MPDL3280A. Now that my analysis revealed a relationship between Hedgehog signaling and immune signal transduction, it may be tempting to combine modulators of Hedgehog signaling with checkpoint-directed therapy.

I propose a sonic hedgehog-initiated novel signaling pathway through Src–Rac cascades, which regulates actin cytoskeletal reorganization and endocytosis. In this framework, Smo coheres to and sequesters Rac in its active form when engaged with for instance the pharmacological compound Purmophamine (B) or Src. Smoothened is released from Patched inhibition in the presence of Sonic Hedgehog which is mediated by Gli-dependent transcriptional activity. Other extracellular signals might regulate secretion of Sonic Hedgehog at particular sites and times. Remarkably, Src and histamine have been shown to trigger Sonic Hedgehog secretion[53], inducing Ca^{2+} influx in the endothelial cells[54]. Suggesting the Sonic Hedgehog secretion in the fibroblast cells due to the Ca^{2+} influx. Calcium has repeatedly been reported to induce expression of Hedgehog ligands. It was reported that Ca^{2+} chelation inhibits Indian Hedgehog gene expression in chick chondrocytes [55]. We describe the impact of subcellular localization of Smoothened on its signaling ability and its chemotactic signaling capacity through a non-canonical pathway, and I feel small mutations in Smoothened may cause its activation in various cancers (but probably not gastric cancer). Importantly, pharmacological activation of Smoothened will activate immune relevant signaling and thus support anti-cancer immunity.

My thesis also contributes to gastric cancer prevention. Gastric atrophy is thought to be a precursor for malignant neoplasm formation. Therefore, enhancements in techniques used for atrophy diagnosis might help identifying individuals at risk for gastric cancer. My findings have thus relevance for recommendations with respect to the examination and grading of atrophy. This will envisage in-depth histologic

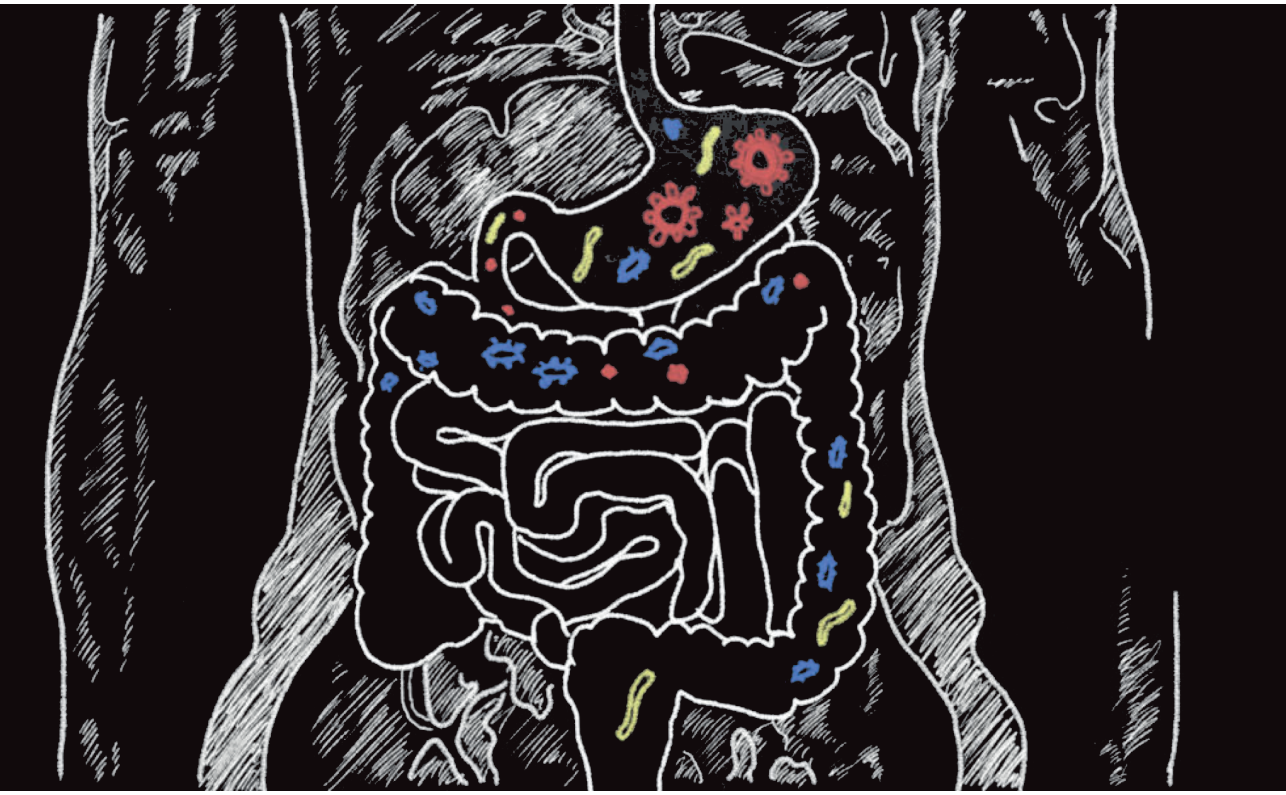
atrophy and should function as a useful assessment tool for evaluation of premetastatic tumor conditions during an endoscopic investigation in routine clinical practice, particularly for patients in developing countries.

Future Perspectives

Hence the former, it seems expedient to restrict the experimental use of Hedgehog inhibitors to those gastric cancer patients that show exaggerated Hedgehog signaling. This is of relevance because currently multiple trials using Vismodegib for the treatment of gastric cancer are being conducted. Although as yet unpublished results indicate that using Hedgehog inhibitors for gastric cancer in unselected patients is not useful, whereas using Vismodegib in selected patients seems to yield better results. And thus, the use of Vismodegib for gastric cancer can serve as a powerful example of the necessity to further develop precision medicine.

Accumulating preclinical as well as clinical studies has shown that the efficacy of checkpoint inhibition could be amplified through dual blockade or in combination with other immunotherapies such as cancer vaccines or adoptive cell therapy. As such, it is worth combining various immune reagents to achieve a maximal effect. Moreover, it is possible that further applications of this approach to traditional chemotherapy or radiation therapy could produce better clinical responses in patients with gastric cancer.

Future studies should clarify the extent to which this novel signaling cascade is involved in other Sonic Hedgehog functions during development. For future research, we recommend an inter-observer agreement done in routine clinical practice among H. pylori-associated gastritis. Parietal cell antibodies should be measured in patients with autoimmune gastritis. Thus in conjunction I feel my thesis opens many novel avenues for future research aimed at better prevention and treatment of gastric cancer. In Conclusion, I feel my thesis opens many novel avenues for future research aimed at better prevention and treatment of gastric cancer.



Chapter 9

Nederlandse samenvatting

Ph.D. Portfolio

List of Publications

Acknowledgments

About the author

Samenvatting voor de leek

Kanker is een ziekte die gekenschetst wordt door de volgende kernprincipes: er zijn cellen die zich ongecontroleerd delen en hier niet mee stoppen; de woekerende cellen verspreiden zich naar het omliggend weefsel en veroorzaken hier schade (infiltratie of invasieve groei); de doorwoekerende cellen verplaatsen zich uiteindelijk ook naar verder weg gelegen plaatsen in het lichaam (uitzaaiing ofwel metastasering). De ziekte is de basis van onnoemelijk veel menselijk leed en met dit promotie-onderzoek heb ik geprobeerd een bijdrage te leveren aan het gevecht der mensheid tegen deze aandoening.

Ik concentreer mij hierbij op maagkanker. De maag vormt een belangrijk onderdeel van het spijsverteringskanaal. De binnenzijde der maag is bedekt door een dikke slijmvlieslaag. Kliertjes binnen dit maagslijmvlies maken het maagsap. In het maagsap zit onder andere zoutzuur maar ook de spijsverteringsenzymen die nodig zijn voor de vertering van de maaltijd. Het zoutzuur is belangrijk voor het doden van de bacteriën welke we middels de voeding binnenkrijgen. Ook activeert zoutzuur de in maag aanwezige spijsverteringsenzymen. Maagkanker is oncologische ziekte uitgaande van de maag. In Nederland zijn er ongeveer tweeduizend gevallen per jaar van maagkanker. Daarmee heeft Nederland een relatief lage incidentie, zowel binnen de Europese Unie alsook mondiaal. Maagkanker verdeelt zich echter niet simpel via sociaal-economische lijnen: in Nigeria is de incidentie bijvoorbeeld ook laag. Ofschoon de vooruitzichten bij vroegtijdige opsporing van maagkanker zeker niet slecht zijn, is helaas zulke vroegtijdige opsporing eerder uitzondering dan regel. Het orgaan zit diep in ons lichaam, een groeiende tumor is niet zichtbaar en geeft ook niet snel pijnklachten. Wanneer duidelijke symptomen manifest zijn is het dan ook vaak te laat. In dit proefschrift probeer ik meer inzicht te krijgen in zowel het ontstaan van maagkanker en een aanzet te geven tot betere behandeling.

In dit opzicht moet eerst de bacterie *Helicobacter pylori* genoemd worden. *Helicobacter pylori* ('spiraalvormige bacterie van de maagportier') is een Gram-negatieve bacterie die in de maag van mensen voorkomt. Deze bacterie, die gedijt in de extreme omgeving van het maagzuur, lijkt verantwoordelijk te zijn voor het ontstaan van maagkanker. Dit gebeurt middels een goed-gekarakteriseerd proces waarbij eerst ontsteking van het maagslijmvlies ontstaat, dat op een gegeven moment uitmondt in een verandering van het maagslijmvlies in een slijmvlies met duidelijke darmkarakteristieken waarna het weefsel een dysplastisch karakter krijgt

en uiteindelijk verwordt tot maagkanker. Echter vaak leidt een infectie met *Helicobacter pylori* niet tot maagkanker. Het blijkt dat verschillende stammen van de bacterie verschillende effecten hebben op de maag in dit opzicht. Een theoretische analyse in dit opzicht geef ik in **Hoofdstuk één** terwijl ik in **Hoofdstuk vijf** hier actief onderzoek naar doe. In dit laatste hoofdstuk analyseer ik zogenaamde plasmiden van de bacterie. Een plasmide is een cirkelvormige streng DNA die zich buiten het chromosomaal DNA bevindt van sommige eencellige organismen, zoals *Helicobacter pylori.* Door analyse van deze plasmiden in patiënten met en zonder ontwikkeling van maagzweer in maagkanker kon ik plasmiden identificeren die geassocieerd zijn met bescherming tegen het ontwikkelen van maagkanker. Het resultaat van deze analyse werd gepubliceerd in het vaktijdschrift Canadian Journal of Gastroenterology and Heptology (Dadashzadeh K, Peppelenbosch MP, Adamu AI. *Helicobacter pylori* Pathogenicity Factors Related to Gastric Cancer. **Can J Gastroenterol Hepatol**. 2017:7942489. doi: 10.1155/2017/7942489) Het bepalen van dergelijke plasmiden in patiënten met *Helicobacter* kan dus van nut zijn om de klinische strategie vast te stellen waar de patiënt de minste overlast (een onderzoek van de maag op het ontstaan van maagkanker middels endoscopie is tamelijk belastend) en het meeste profijt heeft.

Volgend op bacteriële infectie treedt er ontsteking van de maagwand op. Bij een chronische maagslijmvliesontsteking door infectie met *Helicobacter pylori* kan slijmvlies van de maag permanent veranderen. Hierbij wordt het slijmvlies dunner. We noemen dit atrofische gastritis genoemd (atrofie is het verschrompelen van het weefsel). Bij atrofische gastritis wordt het slijmvlies van de maag erg dun, en verliest het bijna alle maagzuur en enzym-producerende cellen. Men kan de diagnose van atrofische gastritis vermoeden gebaseerd op de typische klachten van de patiënt, maar voor definitieve diagnose is een gastroscopie noodzakelijk. Bij zulk onderzoek kijkt de gastro-enteroloog middels een endoscoop (een flexibele slang) via de mondopening en de slokdarm in de maag. Tijdens een maagslijmvliesontsteking oogt het slijmvlies van de maag beschadigd en kan een verdere bevestiging van de diagnose worden verkregen. Voor definitieve diagnose kan de arts tijdens een gastroscopie hapjes weefsel (biopten) uit de maagslijmwand nemen. De patholoog onderzoekt deze biopten dan met de microscoop, en stelt de diagnose dan met zekerheid (of juist niet). In **hoofdstuk zes** onderzoek ik in welke mate voorlopige en definitieve diagnose met elkaar overeen komen. Dit blijkt heel mooi te zijn. Deze informatie kan met name belangrijk zijn voor de Derde Wereld waar de aanwezigheid van de mogelijkheden om pathologisch onderzoek te doen

niet vanzelfsprekend is. Deze data werden wereldkundig gemaakt middels een publicatie in een vaktijdschrift (Adamu Ishaku Akyala, Kianoosh. Dadashzadeh, Alaba Ovye, David Ishaleku, Maikel P. Peppelenbosch. *Agreement between Endoscopic assessment and Histological Diagnosis of helicobacter Pylori−Associated Gastric Atrophy and Intestinal Metaplasia.* **ARC Journal of Hepatology and Gastroenterology** Volume 2, Issue 2, 2017, PP 12).

Een langdurige maagslijmvliesontsteking kan veranderingen in het weefsel veroorzaken en op termijn ontaarden in maagkanker. In dit proefschrift richt ik mij op de onderliggende mechanismen en met name op het ontwikkelingsbiologische hormoon Hegdehog. In een literatuurstudie toon ik aan dat dit Hedgehog een belangrijke rol heeft bij het ontstaan van atrofische gastritis (**Hoofdstuk twee**). Deze analyse naar de rol van Hedgehogsignalering werd voor publicatie geaccepteerd in een vooraanstaande vaktijdschrift (Adamu Ishaku Akyala en Maikel P. Peppelenbosch. Gastric cancer and Hedgehog signaling pathway: emerging new paradigms. **Genes & Cancer** (impact factor 5,39.)., Vol. 9 (1-2), January 2018). Een volgende studie gaat verder experimenteel de diepte in wat betreft Hedgehogsignalering (**hoofdstuk vier**) en karakteriseert op gedetailleerd moleculair niveau de processen die behoud van het kankerstamcelcompartiment door Hedgehog-productie bewerkstelligen ("*Smo-dependent and independent pathways in non-canonical Hedgehog signaling* "). Deze studie mag na revisie opnieuw worden aangeboden aan het prestigieuze tijdschrift **iScience** van Cell press.

Uiteindelijk monden de boven beschreven processen uit in maagkanker. Deze ziekte is moeilijk te behandelen omdat hij slecht reageert op conventionele chemotherapie. Niet uitgezaaide ziekte kan chirurgisch goed behandeld maar bij metastase is zulks zinloos. Een mogelijkheid is echter het lichaam te helpen bij het bestrijden van kanker. Dit kan door het geven van immunocheckpointinhibitoren. Kankers weten vaak aan de reinigende werking van het immuunsysteem te ontkomen door moleculen te maken die ons immuunsysteem remmen. Deze zogenaamde immunocheckpoints zijn belangrijk om te voorkomen dat auto-immuunziekte ontstaat. Een methode om oncologische ziekte te bestrijden is het geven van antilichamen aan de patiënt die deze immunocheckpoints remmen. Het gevolg is dat de immuniteit versterkt wordt maar dit gaat vaak wel ten koste van bijwerkingen door auto-immuniteit. In **hoofdstuk drie** analyseer ik de mogelijkheden van dergelijke immunocheckpointinhibitoren bij de behandeling van maagkanker. Het lijkt er op dat ze beperkt effectief zijn en dat of de patiënten geselecteerd moeten

worden die het meeste baat hebben bij behandeling (iets waarvan ik concludeer dat dat vooralsnog niet mogelijk lijkt) of dat de werking van dergelijke middelen versterkt moet worden. De studie werd bij het ter perse gaan van het proefschrift gereviewed door het vaktijdschrift **Canadian Journal of Gastroenterology and Heptology**

Dit laatste aspect (verdere stimulering van immuniteit) werk ik verder uit in **hoofdstuk zeven**. Het vinden van nieuwe immuunsysteem stimulerende behandelingen is één van de voornaamste uitdagingen in het contemporaine biomedische onderzoek in dit opzicht. We redeneerden dat als we weten wat het immuunsysteem remt, haar inhibitie therapeutisch potentieel zou hebben. Gedurende ruimtevlucht raakt het immuunsysteem onderdrukt en als we weten hoe dat gebeurd zouden we ook de betrokken processen kunnen remmen en daarmee juist het immuunsysteem in haar algemeenheid en antitumor immunologie in het bijzonder, kunnen stimuleren. We willen dus uitzoeken welk mechanisme ten grondslag ligt aan de ruimtevlucht-afhankelijke remming van het immuunsysteem . Een mogelijkheid is immuuncellen gedurende een ruimtevlucht met bacteriële bestanddelen te activeren en vervolgens de moleculaire processen die hierop volgen te vergelijken met deze op aarde. We analyseerden de data die uit dergelijke experimenten kwamen en we kwamen tot de conclusie dat één van de biochemische processen die we gevonden hadden in ons hedgehogonderzoek (de zogenaamde Rac/Rho signalering) hierbij belangrijk was. Hiermee lijken verschillende elementen uit dit promotieonderzoek op het zelfde spoor te komen en worden de contouren hoe verder onderzoek moet plaatsvinden om maagkanker succesvol te bestrijden duidelijk. Deze toekomstvisie geef ik mijn **hoofdstuk acht**. Hiermee komt het proefschrift tot een einde. Ik hoop dat het een bijdrage levert aan een wereld zonder sterfte aan maagkanker.

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Adamu Ishaku Akyala was born in Akwanga of Nasarawa State, Northcentral Nigeria, on 17th March 1982. He was raised by his beloved parents Very Rev and Mrs. Adamu Akyala of the Dutch Reformed Nigeria Church. He obtained his bachelor in Microbiology from the University of Jos. Plateau State. He proceeded to Jos University Teaching Hospital (JUTH) for his associate and Fellows in Biomedical Lab sciences where he was certified as a Biomedical Lab scientist. He obtained two master's degree from the Federal University of Agriculture, Makurdi (Msc. Medical Microbiology) and Benue State University, Makurdi (M.sc Public Health Management) in 2010 and 2014 respectively. In 2016, he began his Ph.D. research programme on "Helicobacter pylori and Gastric Cancer: From Tumor microenvironment to Immunotherapy" at the Laboratory of Gastroenterology and Hepatology, Erasmus MC University Medical Center Rotterdam, the Netherlands. This project under the supervision of Prof. dr. M.P. Peppelenbosch and Dr. A. P. Verhaar. Upon completion of his programme, he is resuming as a senior lecturer at the Department of Microbiology, Nasarawa State University. Keffi Nasarawa State.

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