OVARIAN GRANULOSA CELL TUMORS
HISTOPATHOLOGY, IMMUNOPATHOLOGY AND PROGNOSIS

(Granulosacel tumoren van het ovarium)
(Histopathologie, immunopathologie en prognose)

PROEFSCHRIFT

Ter verkrijging van de graad van
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aan de Erasmus Universiteit te Rotterdam
op gezag van de rector magnificus
Prof. Dr. A.H.G. Rinnooy Kan
en volgens besluit van het college van dekanen.
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"From the unreal lead me to the real
From the darkness lead me to light
From death lead me to Eternal life."

Upanishad

To Dev
Sangeeta and Gaurang

To my mother and
to the memory of my father.

CONTENTS

CHAPTER 1

GENERAL INTRODUCTION

Granulosa cell tumors (GCT) of the ovary are composed of granulosa cells with or without an admixture of theca cells. This rare tumor belongs to the category of sexcord-stromal tumors. All GCTs inspite of their indolent course have to be regarded potentially malignant as the recurrence may occur after as long as 20 years. Except for the presence of extra-ovarian spread other prognostic factors remain debatable.

Due to the uncertainty and disagreement concerning prognostic factors (Young & Scully, 1984; Fox, 1985), this retrospective study was undertaken to try and identify risk factors. Therefore the significance of prognostic indicators such as age, stage, tumor diameter, rupture, histological growth pattern, atypia and mitotic activity has been re-evaluated in this series.

Aneuploidy has been associated with poor prognosis in several types of malignant tumors including ovarian carcinomas (Friedlander et al., 1983). Nuclear DNA flow cytometry was performed on this group of tumors to assess if ploidy was related to prognosis and if this would help in predicting the outcome of these patients.

Inspite of the characteristic histological appearance described in most GCTs it appeared sometimes to be difficult to differentiate them from undifferentiated carcinoma, a poorly differentiated Sertoli-Leydig cell tumor, carcinoid, endometrioid stromal sarcoma or a small cell carcinoma. Considering the much better prognosis of GCT it is important to differentiate this tumor particularly from an undifferentiated carcinoma. The value of tissue specific proteins and tumor markers has been investigated.

Due to the interesting functional aspects of this tumor (predominantly feminising, occasionally virilising) and on the basis of recent work on immunohistochemical localisation of steroids (Kurman, Goebelsmann & Taylor, 1979) an attempt has been made to correlate immunohistochemical localisation of steroids with ultrastructure and clinical manifestations.

This study comprises exclusively of adult type of granulosa cell tumors

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CHAPTER 2

REVIEW OF LITERATURE

Historical Review

A description of the ovarian tumor now named as granulosa cell tumor of the ovary in the literature was first documented by C. von Rokitansky in 1859.

In 1895 C.V. van Kahlden published in the Centralblatt für Algemeine Pathologie und Pathologische Anatomie an accurate histological description under the name "adenoma of the Graafian follicle", a type of neoplasia which resembles a cylindroma in places, containing folliculoid structures elsewhere.

In 1901 H. Schroeder used the term "folliculoma" to describe a tumor reproducing granulosa cells with follicular rosettes. He believed it arose from follicular epithelium and noted endometrium hyperplasia but saw no cause and effect link between these two observations.

In 1907 A. Blau published a very well documented case of granulosa cell tumor. In 1910 Lecene (cited by Varangot, 1937) examining an operative specimen used the term "folliculome lipidique" to describe the first French case of granulosa tumor.

In 1914 F. von Werdt coined the term "granulosa zell tumor" but used this to cover two tumors nowadays diagnosed as granulosa tumors, three Brenner tumors and one ovarian seminoma.

1915 saw the first complete and detailed histological description published by R. Meyer.

In 1922 R. Schroeder published a very detailed case in which endometrial hyperplasia had caused metrorrhagia. He considered that the influence of the tumor was similar to that of persistent follicle and that granulosa cell tumors could create a state analogous to that of "metropathia hemorrhagica". This was thus historically the first case in which the functional influence of the tumor was reported.

In 1925 R. Meyer (cited by Schiller, 1934) reported 4 cases of granulosa cell tumor in postmenopausal women accompanied by

endometrial hyperplasia and metrorrhagia. He assumed that a specific stimulus coming from these tumors was responsible for the uterine changes.

The next important publication was in 1931 by Habbe (cited by Schiller, 1934) describing 33 cases with overall view of the question.

During the following years a series of publications followed. In Germany Neumann in 1933 (cited by Schiller, 1934), in Austria Schiller (1934) and in the United States Novak & Brawner (1934) provided important contributions to the clinicopathological study of granulosa tumors.

Important in the histological diagnosis of granulosa cell tumors is the Call-Exner body. Historically it is of interest that in 1875 Emma Call en Siegmund Exner (cited in Roth & Czernobilsky, 1985) described the bodies which bear their names in "Zur Kenntnis des Graafschen Follikels und des Corpus Luteum beim Kaninchen" (Speert, 1958). Emma Call was a graduate of the University of Michigan and Siegmund Exner was director of neurophysiological research institute in Vienna. They had no previous experience with ovarian histopathology and neither had seen a granulosa cell tumor. They considered the structure they observed to be related to the ovum. Befitting Call and Exners original description the follicular rosettes observed by H. Schroeder have appropriately been termed Call-Exner bodies. The significance of the Call-Exner body for the diagnostic pathologist did not become clear until much later.

In 1937 J. Varangot wrote a classical monograph on granulosa tumors. He felt the term granulosa tumor was not entirely satisfactory as it did not emphasize the existence of the hormonal activity of these neoplasms. However he preferred the term granulosa cell tumor to "granulosa Zell Carcinoma" which was then still used by many authors including Novak among others as the latter implied that these tumors are always malignant which was far from the proven fact. He gave the following definition: "the term granulosa cell tumor is used to indicate all tumors for which the cellular prototype represents structures analogous to the cells of the granulosa and which cause somatic changes as a

result of the excess production of female sex hormones". However in 1973 WHO publication titled "Histological typing of ovarian tumors" (Serov, Scully & Sobin) defined a granulosa cell tumor as a tumor of female cell types containing more than a small component of granulosa cells. Granulosa cell tumors being usually estrogenic, may be inactive or rarely androgenic.

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Embryological aspects

The ovary is derived from three different components: the coelomic epithelium, the underlying mesenchyme and the primordial germ cells.

The gonads appear as a thickening of coelomic epithelium on the ventromedial aspect of the mesonephros in an embryo of 4 weeks. At 5 weeks a number of cellular components can be distinguished in the genital ridges. The mesenchyme is covered on the surface by a proliferating layer of coelomic epithelium (Fig. 2.1) (Blaustein, 1982).

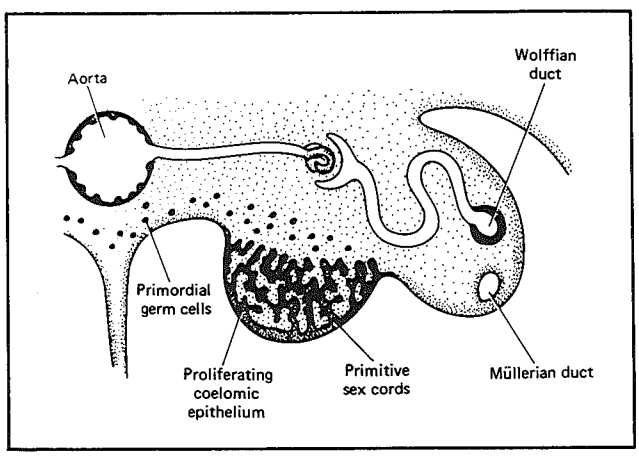

Fig.2-1
Schematic transverse section through the lumbar region of a 6 week embryo, showing the indifferent gonad with the primitive sexcords (A. Blaustein, Pathology of the female genital tract).

At right angles to the coelomic epithelium there are cell condensations in the mesenchyme which are referred to as primitive sexcords. A number of primordial germ cells are present beneath the coelomic epithelium and between the sexcords. By 2 months of gestational age the outer zone of the ovary has formed into ovarian cortex. At 5 months the cortex becomes divided into primitive cortical lobules and cord-like structures (sexcords) by connective tissue septa radiating from the mesenchyme of the medulla. The germ cells which originate in the primitive streak migrate to the ovary and come to be situated in the primitive cortex of ovarian blastema. The germ cells are separated by smaller cells the pregranulosa cells (Fig. 2.2) (Scully, 1979)

encapsulating the individual germ cells to form primordial ovarian follicles.

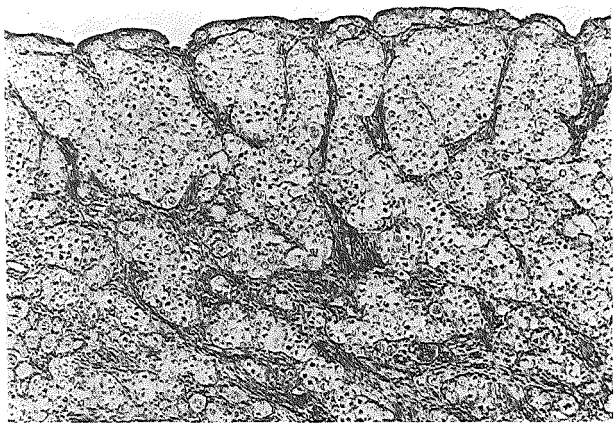

Fig.2-2
Ovary-ovulation age of 5 months. Lobules and cords (sexcords) contain oocytes and pregranulosa cells (R.E. Scully, AFIP, Tumors of the ovary).

Though it is now accepted that mesenchyme of the medulla gives rise to the ovarian stromal cells and their derivatives such as theca cells and luteinised stromal cells, the origin of granulosa cells remains controversial. Some investigators (Gillman, 1948) favour an origin of their precursors, the ovarian sexcords entirely from the down-growths of the coelomic epithelium. Pinkerton et al. (1961) favour the origin of sexcords from the mesenchyme. Still others (Gruenwald, 1942; Jirasek, 1971) conclude that the gonadal blastema is so undifferentiated that it cannot be classified as epithelium or mesenchyme.

Conclusion and histogenesis of Granulosa Cell Tumor

The granulosa cells differentiate from the primitive sexcords of the indifferent gonad but there remains some dispute as to whether these sexcords are derived from coelomic epithelium or primitive gonadal mesenchyme. Most embryologists favour an origin of sexcords and granulosa cells from coelomic epithelium of the genital ridge. However there is no evidence to link granulosa cell tumor to the surface epithelium of the postnatal ovary.

The histogenesis of granulosa cell tumors is largerly speculative. It has been postulated that these neoplasms originate in the vestiges of the embryonal "granulosa ballen", which remained

dormant for years to give rise to the tumor following some unknown stimulation. Schiller (1940) also subscribed to an embryonic origin but from the multipotential mesenchymal cells which may persist to produce granulosa cell tumor in later life. Teilum (1949) subscribed to the view that although granulosa cell tumors and Sertoli cell tumors are considered to be separate lesions they have histological resemblances and are probably of a common blastemic origin.

Granulosa cell tumors are encountered in children, young women in whom granulosa cells are abundant and in older women in whom they have presumably disappeared. Because granulosa cells are very rarely found in the ovaries of postmenopausal women it has been suggested that the neoplasm at this age may originate from unidentified elements within ovarian stroma. In mice granulosa cell tumors have been produced with low dosage radiation therapy for unrelated diseases, however there is no convincing evidence that the association has been one of cause and effect.

Nevertheless it is widely thought that the granulosa cell tumor develops as a result of stimulation of the tissue anlage from which granulosa cells originate but the stimulus to parent tissue of granulosa cells remains unknown.

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Classification of sexcord-stromal tumors

A classification of ovarian tumors formulated by the World Health Organisation in 1973 (Serov, Scully & Sobin, 1973) is based on the microscopic characteristics of the tumors reflecting the nature and origin of identifiable cell types and patterns of growth.

Epithelial tumors
Sexcord-stromal tumors
Germ cell tumors
Lipid cell tumors
Gonadoblastoma
Secondary (metastatic) tumors
Unclassified tumors

Granulosa cell tumor of the ovary belongs to the category of sexcord-stromal tumors composed of gonadal sexcord and stromal derivatives differentiating in an ovarian direction (granulosa and/or theca cells) or in a testicular direction (Sertoli and/or Leydig cells) (Scully, 1980).

A. Granulosa-stromal cell tumor
   1. Granulosa cell tumor
   2. Tumors in thecoma-fibroma group
      a) thecoma
      b) fibroma
      c) unclassified
B. Androblastomas; Sertoli-Leydig cell tumors
   1. Well differentiated
   2. Moderately differentiated
   3. Poorly differentiated
   4. With heterologous elements
C. Gynandroblastoma
D. Unclassified

Granulosa cell tumors are composed of granulosa cells with or without admixture of theca cells. Occasionally a sexcord-stromal tumor has patterns and cell type intermediate between those of granulosa cell tumor and Sertoli-Leydig cell tumor and may have to be placed in an unclassified category (Young & Scully, 1982). Therefore it seems appropriate to describe shortly the salient morphological features of the sexcord-stromal tumors.

Sexcord-stromal tumors

A. GRANULOSA-STROMAL CELL TUMORS

1. Granulosa cell tumor
   Morphology of this tumor will be reviewed in the following chapter
2. Thecoma-fibroma group
   Tumors forming a continuous spectrum from those composed of cells resembling lipid rich theca cells to those entirely composed of cells resembling fibroblasts producing collagen.
   a. Thecoma (theca cell tumor)
      A stromal tumor predominantly composed of uniform oval or spindle shaped cells with lipid rich cytoplasm resembling theca cells and arranged in interlacing bundles. A collagen producing fibrous component is present in varying amounts. Thecomas can be differentiated from diffuse form of granulosa cell tumor by reticulin stain which demonstrates investment of individual theca cells.
   b. Fibroma
      This tumor is composed of spindle cells producing abundant collagen and arranged in interlacing bundles. Varying degree of intercellular edema and occasionally calcification is present.
   c. Unclassified
      Stromal tumors composed of cells intermediate in type between thecoma and fibroma.

B. ANDROBLASTOMA; SERTOLI-LEYDIG CELL TUMORS

Tumors containing Sertoli and Leydig cells in varying proportions. These tumors have been divided in four categories: well differentiated, of intermediate differentiation, poorly differentiated and with heterologous elements (Serov, Scully & Sobin, 1973; Scully, 1980). The well differentiated tumors are composed of tubules lined by Sertoli cells and separated by Leydig cells. These well differentiated tumors contain abundant lipid in the Sertoli cells. Tumors of intermediate differentiation show immature Sertoli cells arranged and separated by clusters of Leydig cells in the stroma. The cells of poorly differentiated tumors are usually spindle shaped and these tumors may resemble a spindle cell sarcoma. Tumors of intermediate or poor differentiation may contain in addition heterologous elements of endodermal derivation such as mucinous epithelium of gastrointestinal type containing goblet cells; argentaffin cells may also be encountered in epithelial lining. Mesodermal elements identified include skeletal muscle, cartilage, bone, fat and smooth muscle.

C. GYNANDROBLASTOMA

Exceptionally rare tumor composed of collections of granulosa cells with Call-Exner bodies and hollow tubules lined by Sertoli cells.

D. UNCLASSIFIED

Tumor with sexcord and/or stromal elements which can not be identified as male or female type. This category includes rare "sexcord tumor with annular tubules", commonly multiple and associated in about one third of cases with Peutz-Jeghers syndrome, gastrointestinal polyposis with oral and cutaneous melanin pigmentation. Its distinctive morphological appearance is characterised by simple and complex ring shaped tubules containing cells of Sertoli and granulosa type, presence of hyaline bodies and calcification.

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Young RH, Scully RE. Ovarian sexcord-stromal tumors. Recent progress. Int J Gynecol Path 1982;1:101-123.

Morphology of Granulosa Cell Tumors and Differential Diagnosis

Granulosa cell tumors (GCT) are composed of granulosa cells with or without theca cells in their stromal component.

Macroscopical appearances

The granulosa cell tumors are frequently but not always encapsulated and have a smooth or lobulated surface. The neoplasms may vary greatly in size from a microscopical lesion which is detectable only histologically to a huge mass over 30 cm in diameter. The diameter usually varies from 5-15 cm (Stenwig, Hazelkamp & Beecham, 1979).

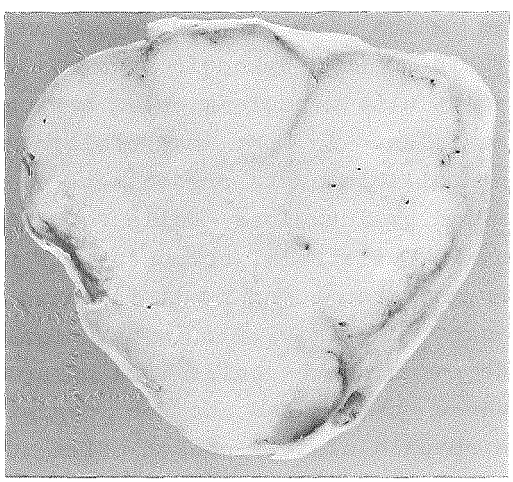

Fig.2-3
A predominantly solid granulosa cell tumor.

Although majority of the tumors are solid (Fig. 2.3) a substantial proportion are partially cystic and some are totally cystic, rarely appearing as a large thin walled cyst resembling a serous cystadenoma (Norris & Taylor, 1969). The solid tumors may be grey to yellow with areas of haemorrhage or necrosis. Multicystic tumors may contain watery fluid or more often blood.

Microscopical appearances

The tumor is composed of small round, polygonal or spindle shaped cells containing a small amount of cytoplasm and rather indistinct boundaries. The nuclei may be round, ovoid or angular with a well defined limiting membrane, longitudinal grooves, resulting in coffee-bean appearance. A single small nucleolus is usually present.

Mitosis range from rare to numerous. The tumor may or may not show a stromal component of fibroblasts and theca cells and either cell types may be luteinised. A variety of microscopic patterns of growth may be encountered with 2 or more patterns commonly coexisting in the same specimen. These include microfollicular (Fig. 2.4), macrofollicular (Fig. 2.5), trabecular (Fig. 2.6), insular (Fig. 2.7) and diffuse (sarcomatoid) (Fig. 2.8). The microfollicular pattern is characterised by the presence of Call-Exner bodies which are small rounded spaces containing eosinophil material in which pyknotic nuclei and nuclear fragments may be present with granulosa cells arranged non-uniformly around these cavities. In the macrofollicular pattern large cysts resembling follicle cysts predominate. The trabecular pattern is characterised by granulosa cells arranged in anastomosing cords or ribbons in the stromal component. If the granulosa cell cords are narrow and stroma scanty it is known as the watered silk or moire silk pattern (Fig. 2.9). In the insular pattern tumor cells form islands in which the cells lack polarity except those at the margins where they tend to be radially arranged. The diffuse pattern reveals closely packed cells in a diffuse arrangement.

GCT usually contains small lipid droplets (Fig. 2.10) but when partially luteinised, they become heavily lipid laden. Reticulin stain reveals sparse fibrils which tend to delineate groups of cells rather than individual cells. (Fig. 2.11).

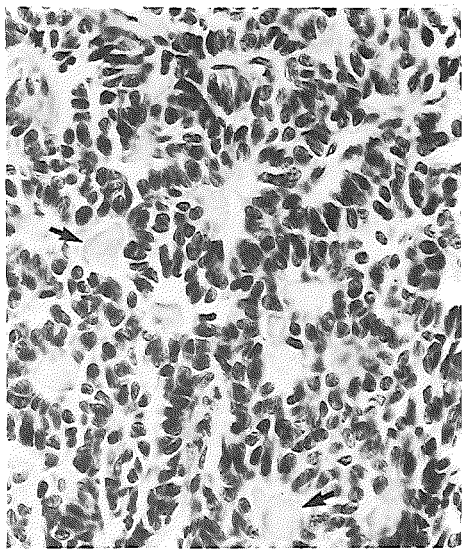

Fig.2-4
A granulosa cell tumor showing microfollicular pattern with Call-Exner bodies (arrows). (H&A x380)

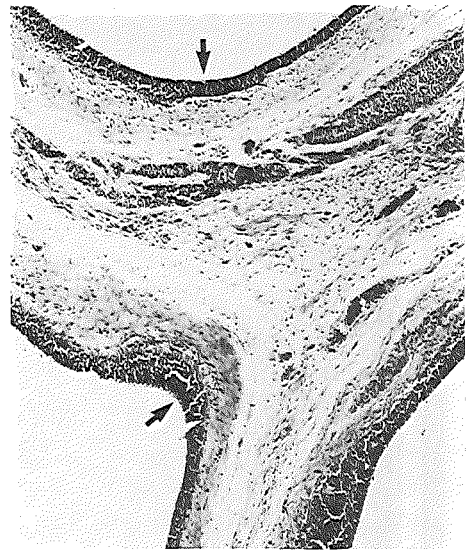

Fig.2-5
Macrofollicles (arrows) in a granulosa cell tumor. (H&A x150)

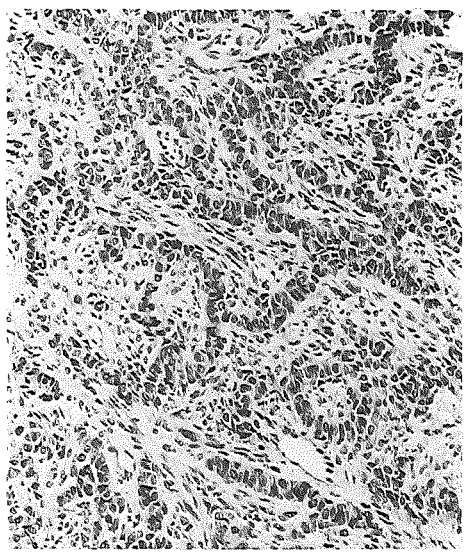

Fig.2-6
Granulosa cell tumor showing a trabecular pattern. Trabeculae of granulosa cells are separated by thecofibromatous stroma. (H&A x150)

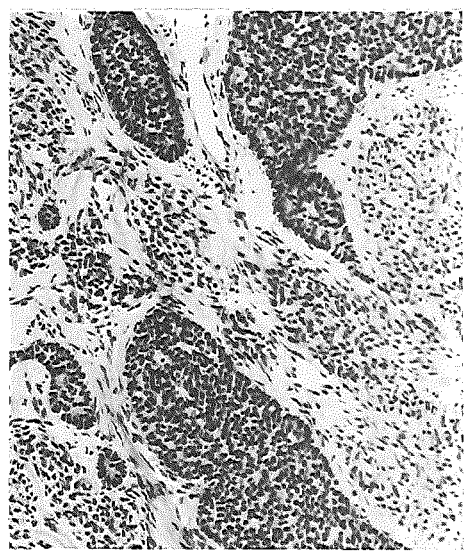

Fig.2-7
An insular pattern in granulosa cell tumor. (H&A x150)

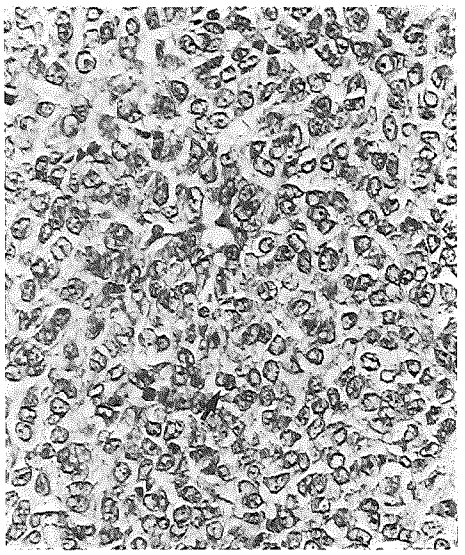

Fig.2-8
A diffuse arrangement of cells in granulosa cell tumor. Occasional cells show a nuclear groove (arrow). (H&A x380)

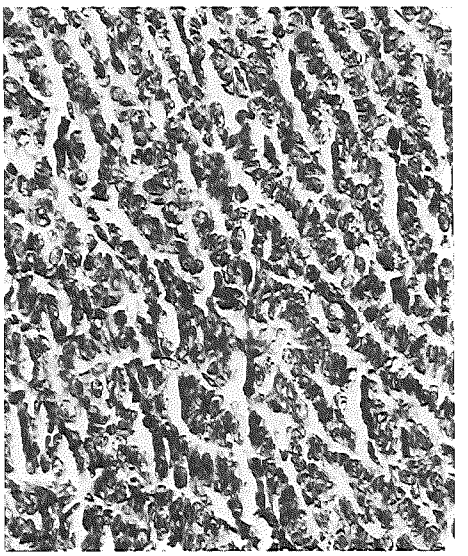

Fig.2-9
A "moire" silk or "watered" silk pattern in a granulosa cell tumor. (H&A x380)

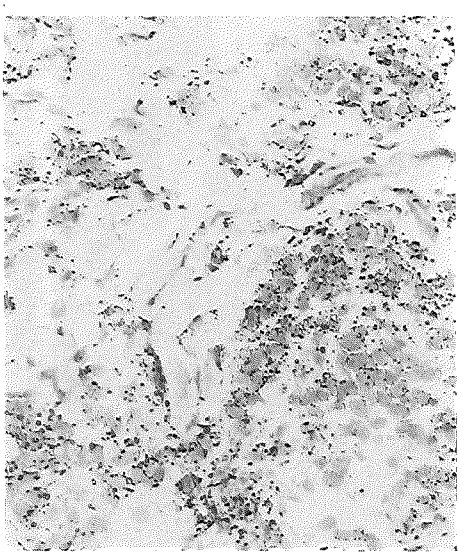

Fig.2-10
Lipid droplets in granulosa theca cells. (ORO stain x150)

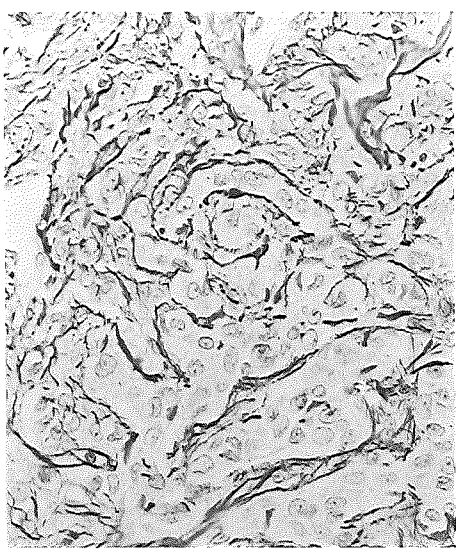

Fig.2-11
Reticulin stain showing fibrils delineating groups of cells in a granulosa cell tumor. (Gomori x380)

Differential diagnosis

The precise diagnosis of GCT is of critical importance because in general the management of these neoplasms is at great variance with that of the common epithelial tumors of the ovary in as much as GCTs show a more "benign" biological behaviour and better prognosis than others (Fox, Agrawal & Langley, 1975).

Undifferentiated carcinomas, poorly differentiated adenocarcinomas and carcinoids are commonly misinterpreted as GCT on microscopic examination. Some undifferentiated carcinomas may form areas with included spaces (Fig. 2.12) resembling Call-Exner bodies but carcinomas typically show hyperchromatic often pleomorphic nuclei with frequent mitosis (Fig. 2.13) in contrast to the pale grooved nuclei with fewer mitosis in GCTs.

A primary small cell carcinoma of uncertain nature occurring in young women and often associated with paraneoplastic hypercalcemia may be confused with GCT (Dickersin, Kline & Scully, 1982). It is important to distinguish between this tumor and GCT as former is associated with a poor prognosis. Microscopically a small cell carcinoma shows small closely packed epithelial cells with scanty cytoplasm and small dark nuclei possessing prominent nucleoli usually arranged diffusely or in islands, cords or trabeculae. Mitosis are common and nuclear grooves are absent (Fig. 2.14).

The carcinoid tumor must be distinguished from GCT as they also show insular (Fig. 2.15), trabecular and acinar patterns (Fig. 2.16) (Scully, 1979). Gland formation, round nuclei with coarse chromatin and luminal calcific deposits are of diagnostic aid but the most specific finding is a cytoplasmic content of argentaffin or argyrophil granules (Fig. 2.17).

Distinction between GCT and **Sertoli-Leydig cell tumors** depends on recognition of differentiation in the direction of ovarian or testicular structures. Sertoli cells are often arranged in tubules, cords or diffuse patterns (Fig. 2.18) in contrast to the microfollicular trabecular, insular or diffuse arrangement of granulosa cells. Leydig cells (Fig. 2.19) tend to cluster while lutein cells cluster less often. Twenty percent of

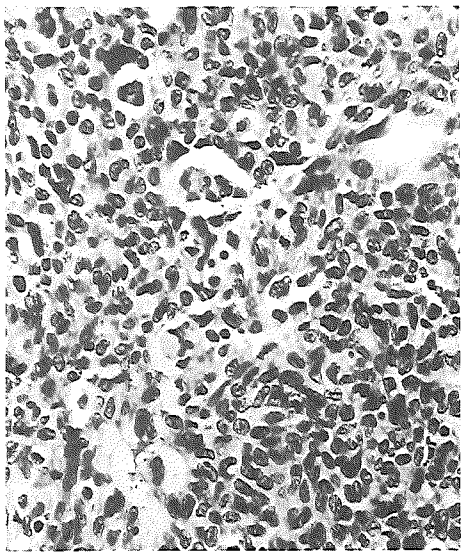

Fig.2-12
Undifferentiated carcinoma showing small included spaces. (H&A x380)

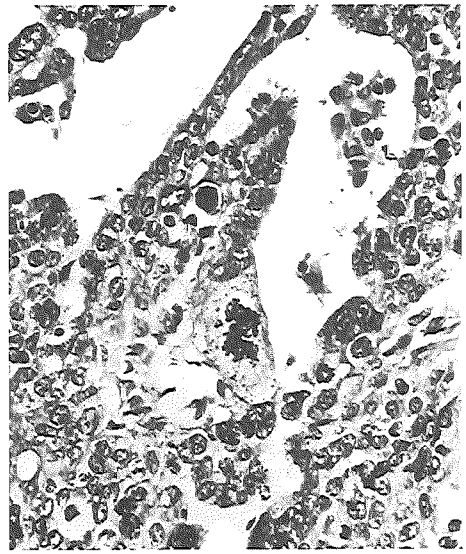

Fig.2-13
Higher magnification of Fig.2-12 showing hyperchromatic nuclei and mitosis.

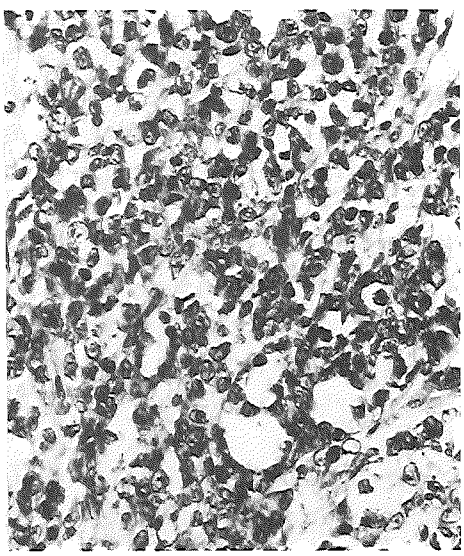

Fig.2-14
Small cell carcinoma with diffuse arrangement of closely packed cells with dark nuclei. (H&A x380)

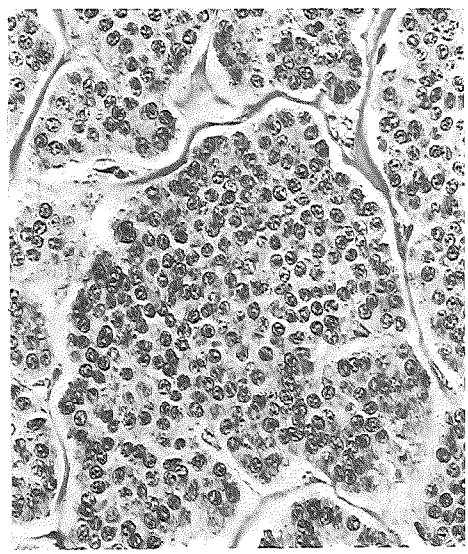

Fig.2-15
Carcinoid tumor with insular arrangement of cells. (H&A x380)

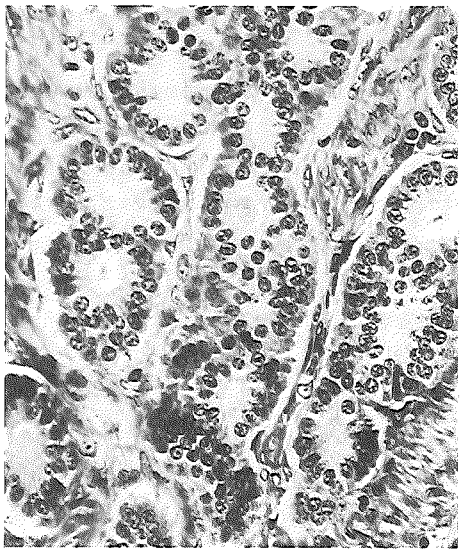

Fig.2-16
Another area of carcinoid tumor in (Fig.2-15) showing acinar pattern. (H&A x380)

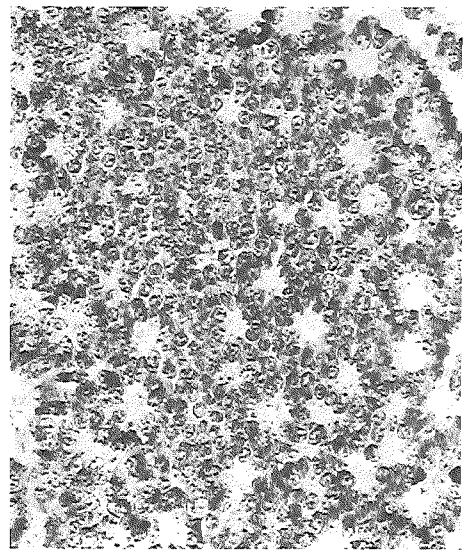

Fig.2-17
Carcinoid tumor showing Grimelius positive granules. (x380)

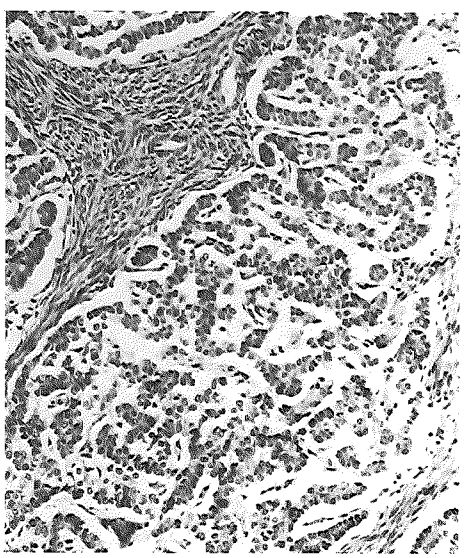

Fig.2-18
Sertoli-Leydig cell tumor (SLCT) with cords of Sertoli cells resembling granulosa cell tumor. (H&A x150)

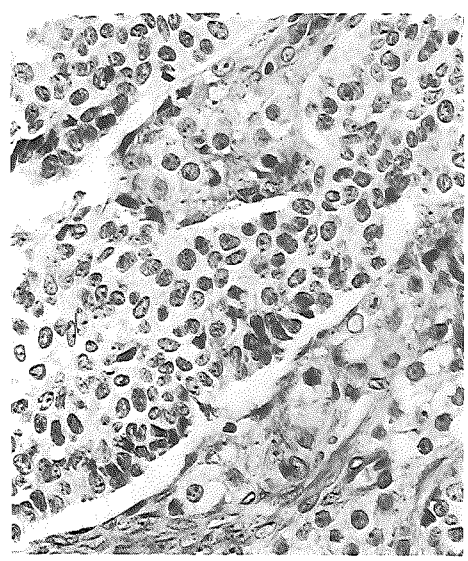

Fig.2-19
SLCT in (Fig.2-18) revealed occasional clusters of Leydig cells. (H&A x380)

Sertoli-Leydig cell tumors contain heterologous elements while in GCT these are absent.

GCTs may be confused with rare **primary endometrioid** or **metastatic endometrial stromal sarcoma** (Fig. 2.20) of the ovary (Young, Prat & Scully, 1982). On microscopic examination a rich network of small arterioles resembling endometrial spiral arterioles is typically present in stromal sarcoma and nuclei lack the grooves characteristic of granulosa cells (Fig. 2.21). A reticulin stain reveals individual cell investment by fibrils in stromal sarcomas.

Other secondary small celled tumors may roughly imitate a GCT. These include a neuroblastoma, lymphoma and in particular a bronchial small cell carcinoma which may form islands, cords and folliculoid spaces of small dark cells suggesting a GCT. These are distinguished by lack of typical granulosa cell areas and neurosecretory granules. See also section Electron Microscopy and Tumor markers (Chapter 2).

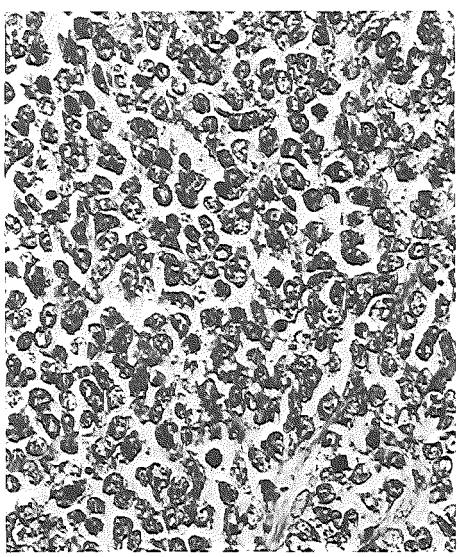

Fig.2-20
Endometrioid stromal sarcoma resembling granulosa cell tumor.
(H&A x380)

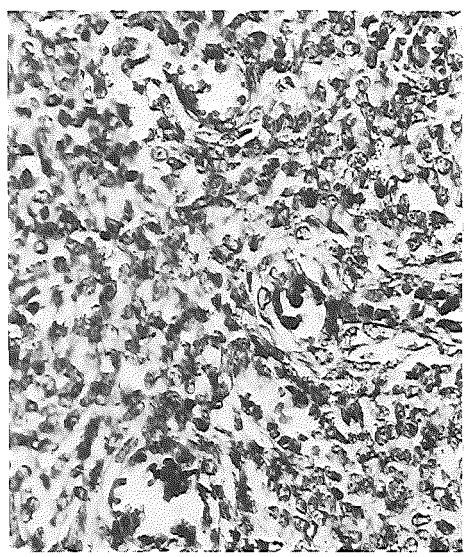

Fig.2-21
Endometrioid stromal sarcoma showing many arterioles and nuclei lacking grooves. (H&A x380)

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Electronmicroscopic features and differential diagnosis

Granulosa cell tumors have a characteristic ultrastructural morphology (Hernando Salazar & Amador Gonzalez-Angulo, 1984; Genton, 1980; Pedersen & Larsen, 1970). Tumor cells are round or polygonal with well defined cell membranes attatched by immature desmosomes. Numerous cytoplasmic projections are visible in the wide intercellular spaces. A basement membrane and occasional collagen fibres surround the tumor cells. The nuclei show a single deep indentation resulting in coffee-bean appearance. Nuclear chromatin reveals peripheral margination. Nucleoli are generally prominent (Fig. 2.22).

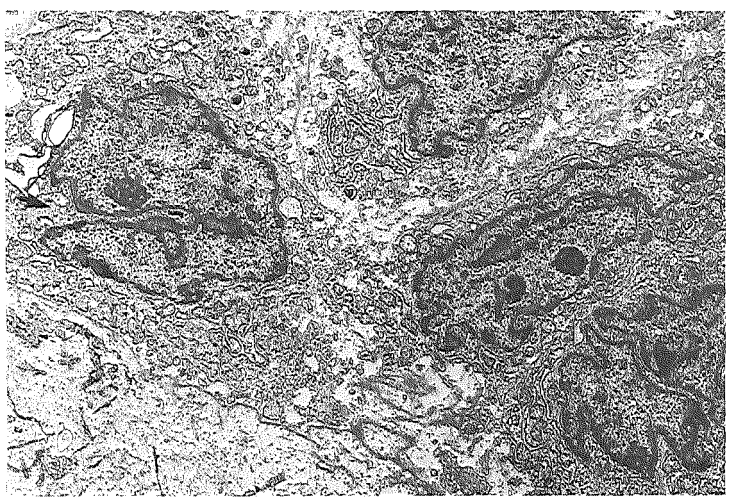

Fig.2-22
Granulosa cell tumor showing coffee-bean nuclei with a deep indentation (arrow) and wide intercellular spaces. x4000

The cytoplasm of non-luteinised tumor cells reveals moderately developed, predominantly granular, endoplasmic reticulum in parallel rows. Dilated cisternae of the granular endoplasmic reticulum are filled with fine granular material. Lipid droplets and lysosomes are scanty. Mitochondria are small with lamellar cristae and numerous microfibrils.

The cytoplasm of luteinised cells is characterised by a well developed tubular agranular endoplasmic reticulum as found in steroid producing cells. Mitochondria display tubular cristae.

Call-Exner bodies are intercellular spaces surrounded by neoplastic cells lying on a basal lamina. These cavities contain amorphous material of moderate electron density, fibrillar structures and cellular detritus.

Besides the above two cell types a few elongated cells of smaller size may be present displaying a dark nucleus, electron dense cytoplasm and numerous swollen mitochondria. These cells resemble non-specialised ovarian stroma cells and may be theca cells.

The precise diagnosis of the granulosa cell tumor is of utmost importance keeping in mind the difference in prognosis and treatment particularly between granulosa cell tumor and undifferentiated carcinoma. Identification of Call-Exner bodies is important in differentiation from undifferentiated carcinoma which, may show small spaces that resemble Call-Exner bodies. Granulosa cell tumors do not form glands or tubules, so no lumina or luminal structures e.g. microvilli, cilia or tight junctions are usually seen. Irregular nuclear pattern with clumped chromatin seen in undifferentiated carcinoma helps to differentiate a granulosa cell tumor with its typical coffee-bean nuclei (Fig. 2.23). Distinction between granulosa cell tumor and carcinoid depends on the presence of uniform rounded nuclei with dispersed chromatin and scanty cytoplasm in the carcinoid. The latter also show neurosecretory granules and positive Grimelius staining (Fig. 2.24).

Sertoli-Leydig cell tumor can be distinguished by characteristic features of Sertoli cells, their form of arrangement and the recognition of Leydig cells (Kooijman & Straks, 1982). Sertoli cells possess irregular angulated outlines, desmosomes and tight junctions. In contrast to the granulosa cell tumor luminal spaces presenting apical microvilli and occasional cilia are found. The cytoplasm of Sertoli cells is rich in organelles and frequently show concentric arrangement of smooth endoplasmic reticulum, mitochondria, well developed Golgi apparatus, scatte-

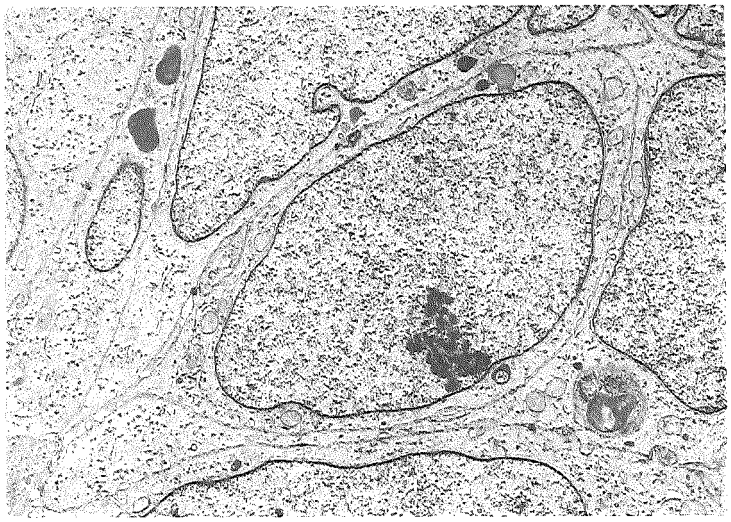

Fig.2-23
Undifferentiated carcinoma showing irregular nuclei without coffee-bean appearance. Cells are attatched by desmosomes. x6000

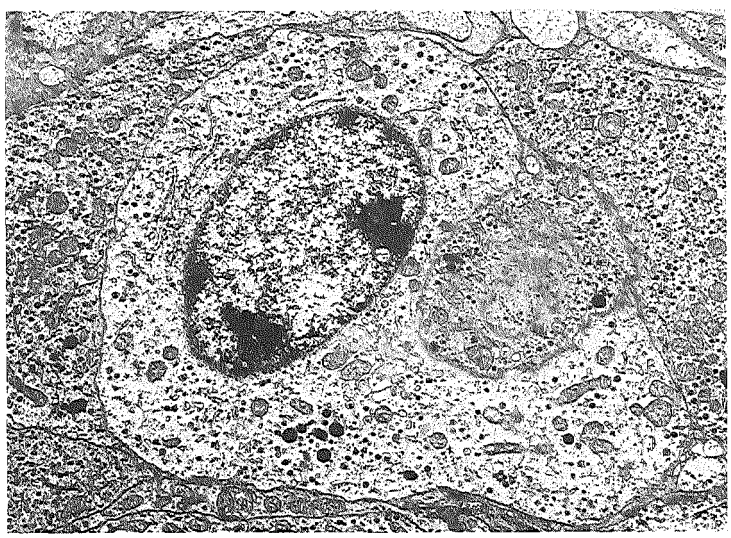

Fig.2-24
Carcinoid tumor, showing epithelial cell with a uniform round nucleus and numerous neurosecretory granules. x6000

red cisternae of rough endoplasmic reticulum and lysosomes. In contrast to the single indentation of coffee-bean nuclei irregular deeply indented and convoluted nuclei with well dispersed chromatin and multiple nucleoli are present. Endometrial stromal sarcoma (Akhtar, Kim & Young, 1975) reveals small polygonal or rounded cells without intercellular attatchment with relatively scanty cytoplasm and central irregular nuclei with well dispersed chromatin and discrete nucleoli. Cytoplasm shows a moderate number of small mitochondria, parallel cisternae of rough endoplasmic reticulum, small groups of lysosomes microfilaments and lipid droplets.

Electronmicroscopy so far failed to reveal any specific features to identify the cell type of ovarian small cell carcinoma with hypercalcemia.

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Tumor markers

Tumor markers can be defined as a substance which is selectively produced by a tumor and released into the circulation in detectable amounts (Van Nagel, 1983). Since there is virtually no reliable method of early diagnosis for ovarian tumors the potential use of tumor markers in ovarian cancer is great. Several types of tumor markers have been described in ovarian cancer e.g. oncofetal antigens, the two most important being carcinoembryonic antigen and alpha-fetoprotein, carcinoplacental proteins such as humanchorionic gonadotrophin, placental alkaline phosphatase, human placental lactogen and tumor associated antigens e.g. CA 125.

At present the practical contribution of tumor markers in ovarian cancer is maximum in germ cell tumors. Unfortunately no tumor marker has been described so far for ovarian sexcord stroma cell tumors. Traditionally estrogen synthesis has been attributed to theca cells, progesterone production to luteinised granulosa cells i.e. those comprising corpus luteum and testosterone synthesis to Leydig cells. Non-luteinised granulosa cells and Sertoli cells have been considered to be inactive. Kurman, Goebelsmann & Taylor, 1979; 1981; Kurman, Ganjei & Nadji, 1984) have described immunocytochemical localisation of steroid hormones in gonadal stromal tumors. The results of their study indicated that in granulosa theca cell tumor granulosa cells were primarily responsible for estrogen production. Judging from the intensity of staining reaction estradiol appeared to be mainly localised in granulosa cells and progesterone in theca cells. They considered the peroxidase-antiperoxidase method sufficiently sensitive to detect the proportion of steroid remaining after tissue processing. However they cautioned that a positive reaction did not necessarily imply steroid synthesis as it could also indicate storage or binding of steroids to specific hormone receptors in cells.

Ovarian sexcord stroma cell tumors have been studied immunohistochemically for the presence of intermediate filament proteins (Miettinen, Lehto & Virtanen, 1983; Miettinen et al.,

1985). They demonstrated the vimentin positivity and keratin negativity of sexcord stromal tumors. Normal granulosa cells also contain vimentin as determined biochemically (Albertini & Kravit, 1981).

Considering a much better prognosis of granulosa cell tumors as compared to the poorly differentiated epithelial cancer from which they may be difficult to differentiate, antibodies to intermediate filaments may be of help in the differential diagnosis. Granulosa cell tumors express vimentin but lack cytokeratin whereas the poorly differentiated ovarian carcinomas express cytokeratin. However in some ovarian carcinomas vimentin positive tumor cells can be found (Miettinen et al., 1983), therefore vimentin cannot be considered an unequivocal marker for mesenchymal cells or mesenchymal tumors.

Our detailed observations on granulosa cell tumors are reported in Chapters 4 and 5. The immunohistochemical marker profile and Grimelius staining results of GCT and tumors to be considered in differential diagnosis are summarised in addendum to chapter 5.

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Clinical features

Granulosa cell tumors account for approximately 1.6% of all ovarian tumors (Hodgson, Dockerty & Mussey, 1945) and 6% of ovarian cancers (Bennington, Ferguson & Haber, 1968).

These neoplasms are usually unilateral with only 5-8% being bilateral. In case of bilateral tumors it is almost impossible to determine whether there are two primary tumors or a primary tumor in one ovary with a metastasis in the other.

Granulosa cell tumors occur mainly in women aged between 30 and 70 years with a peak incidence between 45 and 55 years (Fox & Langley, 1976). Less than 5% of granulosa cell tumors are encountered before puberty (Scully, 1977a). Approximately one third of the granulosa cell tumors occur in the premenopausal patients and the remainder in the postmenopausal age group (Bjorkholm & Silfversward, 1981).

Granulosa cell tumors are the most common ovarian tumors associated with endocrine manifestations. Approximately three fourth of them being associated with hyperestrinism. The nature of these manifestations varies with the age of the patient. In prepubertal girls granulosa cell tumors owing to their estrogen secretion can produce some evidence of sexual pseudoprecocity. Enlargement of breasts is usually the first sign of precosity, the appearance of pubic and axillary hair, the enlargement of secondary sex organs and irregular uterine bleeding being the later manifestations. Vaginal bleeding preceded by white vaginal discharge is almost invariably noted in these girls. Skeletal development may be accelerated.

In premenopausal women most patients with granulosa cell tumor experience a variety of menstrual disorders including menometrorrhagia which may be preceded by oligomenorrhoea and amenorrhoea. These menstrual disorders are related to hyperestrinism leading to hyperplasia of endometrium. Painful enlargement of breasts my be a prominent symptom.

In most cases uterine bleeding is the typical manifestation in postmenopausal women with granulosa cell tumor.

Endometrium in women with functioning granulosa cell tumors reveals a variety of changes ranging from cystic hyperplasia to atypical hyperplasia and adenocarcinoma. Endometrium carcinoma is typically low grade developing in approximately 5% of all women with a granulosa cell tumor (Scully, 1977b) and twice as common after menopause as it is in younger women (Gusberg & Kardon, 1971).

Rarely granulosa cell tumors, often unilocular or multilocular thin-walled cystic tumors, have led to virilisation (Norris & Taylor, 1969; Giuntoli et al., 1976).

In addition to the endocrine manifestations these neoplasms may produce non-specific symptoms of ovarian tumor such as abdominal pain, swelling, backache and dysuria. Abdominal distention may be due to a large tumor or due to ascites which is encountered in about 10% of cases. Very rarely pleural effusion is also present (Meig's Syndrome). The granulosa cell tumor is particularly prone to rupture often being accompanied by hemoperitoneum and resultant acute abdominal symptoms. This complication has been reported in 5 to 20% of cases.

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Prognosis

Granulosa cell tumors possess a low malignant potential reflected by their slow protracted course and infrequent, often late recurrence.

There are no definite and reliable criteria to judge the prognosis in individual patients (Fox, Agrawal & Langley, 1975). The clinical stage is however of prognostic significance. Ninety percent of granulosa cell tumors present as stage I tumors. If it is clinically malignant when first encountered the spread is typically to adjacent pelvic structures. Recurrences which are often seen 5 years or more after primary treatment and occasionally 20-30 years postoperatively also tend to remain localised to pelvis or abdomen. Lymph node metastasis and blood borne metastases to the lungs, liver, bone and brain do occur rarely and sometimes long after initial diagnosis. Stage I tumors have a considerably better prognosis than advanced clinical stages. Stenwig, Hazelkamp & Beecham (1979) and Bjorkholm & Silfversward (1981) in their large series (118 and 198 cases respectively) found a relative survival rate at ten years of 86% versus 49% and 96% versus 26% respectively.

Bjorkholm & Silfversward (1981) found that if in stage I disease the tumor had ruptured spontaneously or during operation it was a bad prognostic sign. Their series (198 cases) showed 86% relative 25 years survival of patients with intact stage I tumors in contrast to 60% survival of those with ruptured tumors in the same stage.

Patients below 40 years of age at diagnosis are reported to have a better prognosis than older patients (Fox, Agrawal & Langley, 1975; Stenwig, Hazelkamp & Beecham, 1979).

However, Bjorkholm & Silfversward (1981) concluded from their survey that age at diagnosis had no influence on relative survival.

Tumor size is related to prognosis as tumor measuring more than 15 cm are indicative of relatively poor prognosis (Fox, Agrawal & Langley, 1975). Their series (92 cases) showed a 100% 5 year survival of patients with tumors 5 cm or less in diameter

but only 64% survival of those with tumors 6 to 15 cm in diameter but 61% survival when the tumor was larger than 15 cm. The 10 year survivals in this series were 100%, 57% and 53% respectively. Stenwig, Hazelkamp & Beecham (1979) in their series (118 cases) reported a crude overall survival of 73% in patients with tumors under 5 cm in diameter, 63% survival with those between 5 and 15 cm diameter and 34% with tumors larger than 15 cm in diameter.

In a series (198 cases) from Radiumhemmet analysed by Bjorkholm & Silfversward (1981) stage I tumors with a diameter less than 5 cm showed a 100% relative 10 year survival as compared to 92% survival for larger than 5 cm stage I tumors but the difference did not reach statistical significance. Thus, the data in the literature did not establish a relationship between tumor size and prognosis independent of stage (Young & Scully, 1984).

Attempts to correlate the morphological features to prognosis have not been uniformly successful. Several investigators (Norris & Taylor, 1968; Fox, Agrawal & Langley, 1975; Stenwig, Hazelkamp & Beecham, 1979; Bjorkholm & Silfversward, 1981) found no relation between the various histological patterns of granulosa cell tumor to prognosis. Kottmeier (1953) reported a significantly poor prognosis with a diffuse growth pattern as compared to the follicular or "Cylindromatous" pattern.

Relationship between mitotic activity of granulosa cell tumors and prognosis has been reported (Fox, Agrawal & Langley, 1975). Stenwig, Hazelkamp & Beecham (1979) found a 70% 10 year survival associated with 2 or fewer mitotic figures per 10 high power fields (H.P.F.) compared to a 37% survival when three or more mitosis were present. Bjorkholm & Silfversward (1981) also found that patients having tumors with great number of mitosis had a significantly worst outcome. However, most of the tumors with higher mitotic rates were also at higher stage than those with a low mitotic rate and the difference in mitotic rate did not have a statistically significant effect on the prognosis of stage I tumors.

Stenwig, Hazelkamp & Beecham (1979) concluded that the degree of cellular atypia seems to be closely correlated to prognosis

with a 5 year survival of 98% when the tumor showed no atypia compared to 85% when there was slight atypia and 33% for those with moderate degree of atypia. Bjorkholm & Silfversward (1981) in their series reported 80% relative survival of 25 years in patients with grade 1 nuclear atypia as compared to a 60% survival in those with grade 2.

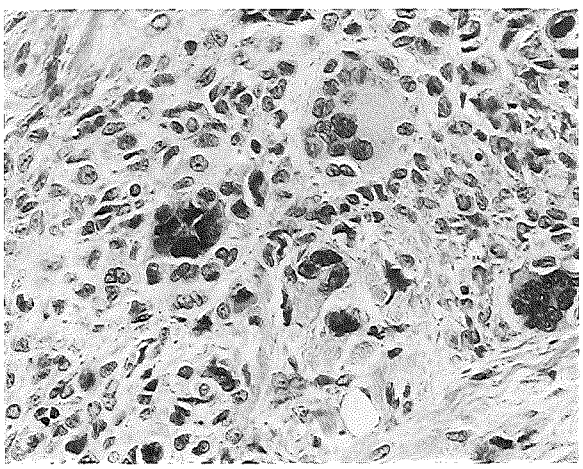

Fig.2-25
Granulosa cell tumor showing mononucleate and multinucleate cells with bizarre hyperchromatic nuclei. (H&A x380)

Approximately 2% of granulosa cell tumors contain mononucleate and multinucleate cells with large bizarre hyperchromatic nuclei (Fig. 2.25) the presence of which does not appear to worsen the prognosis (Young & Scully, 1983). They suggested that these nuclear changes are probably degenerative in nature.

Conclusion: Factors affecting prognosis

From the literature it can be concluded that clinical stage is of considerable prognostic significance as stage I tumors have better prognosis than higher stage tumors.

Rupture of the granulosa cell tumor adversely affects prognosis.

Age above 40 at the time of diagnosis indicated a relatively poor survival rate. The only pathological feature consistently related to prognosis is its size.

Attempts to correlate the histological pattern, mitotic activity and the degree of nuclear atypia with prognosis have not been uniformly successful.

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Treatment

The treatment of granulosa cell tumors depends on the stage of the tumor and the age of the patient. As granulosa cell tumors are bilateral in less than 5% of the cases and 90% present in stage I, unilateral salpingo-oophorectomy is justified in the younger woman who desires to preserve fertility (Morrow & Townsend, 1981). For older and postmenopausal women hysterectomy with bilateral salpingo-oophorectomy is the surgical treatment of choice. High stage tumors and recurrent tumors may respond favourably to radiation therapy (Kalavathi, 1971; Schwartz & Smith, 1976).

There is little reported experience of chemotherapy in granulosa cell tumors but combination chemotherapy with actinomycin-D, 5 fluerouracil and cyclophosphamide has been reported to be effective in a few cases (Schwartz & Smith, 1976). In young patients treated by conservative surgery subsequent pregnancies have been recorded confirming a return to normal fertility.

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CHAPTER 3

PROGNOSTIC FACTORS IN OVARIAN GRANULOSA CELL TUMORS A CLINICOPATHOLOGICAL STUDY

Introduction

Granulosa cell tumors (GCT), which account for less than 2% of all ovarian tumors (Scully, 1979), possess a low malignant potential reflected by a relatively good prognosis, slow protracted course and, if it occurs, a late recurrence. There is a lack of unanimity over the influence of different prognostic factors. However, clinical stage has consistently been related to survival, stage I tumors showing a considerably better prognosis than higher stage tumors (Stenwig, Hazekamp and Beecham, 1979; Bjorkholm and Silfversward, 1981). Also age at onset of diagnosis is reported to have a favourable effect on prognosis i.e. patients below 40 having a longer survival than older patients (Fox, Agrawal and Langley, 1975; Stenwig, Hazekamp and Beecham, 1979). In contrast, Bjorkholm and Silfversward (1981) found no such association. In their series rupture of the tumor adversely affected the outlook.

The only pathological feature of GCT which has been consistently related to prognosis is size (Sjostedt and Wahlen, 1961; Fox, Agrawal and Langley, 1975; Stenwig, Hazekamp and Beecham, 1979; Bjorkholm and Pettersson, 1980; Bjorkholm and Silfversward, 1981). On the correlation of histological pattern, mitotic activity and degree of nuclear atypia with prognosis opinions differ widely.

In this study we describe 61 cases of granulosa cell tumors with special emphasis on the factors influencing prognosis.

Materials and Methods

Hundred and nineteen patients with a primary diagnosis of granulosa cell tumor were reviewed. This material was collected

during 19 years (1966-1985). Hundred and five patients had been registered by the Netherlands Committee for Ovarian Tumors and 14 were referred to the Rotterdam Radiotherapy Institute.

The clinical data from these 119 patients at the time of diagnosis was available. Clinical follow-up of these patients was sought but was successfully obtained in only 76 cases. The autopsy findings when available were reviewed.

After excluding incorrectly diagnosed cases and those from which histopathological material was unavailable 61 cases remained which form the basis of our study. The maximum follow-up period was 19 years. All tumors were classified according to the WHO international histological classification of ovarian tumors (Serov, Scully and Sobin, 1973). In relation to the size of the tumor a sufficient number of slides was available. When required, new sections were cut and stained with hematoxylin azofloxin. The majority of the tumors were pure granulosa cell tumors with in a few a small inconspicuous theca cell component.

The following features were studied for their effect on survival: FIGO (International Federation of Gynecology and Obstetrics) stage at the time of operation, age at diagnosis, size of the tumor, rupture of tumor, histological pattern, and presence of lymphatic invasion and necrosis. Nuclear atypia was evaluated according to the degree of nuclear pleomorphism and hyperchromasia and graded as absent, slight, moderate and severe. Mitoses were counted on an average over 40 to 50 high power fields (H.P.F.) by using a 40 x objective and 12.5 x occulars and expressed per 10 H.P.F. Presence or absence of endometrial hyperplasia and/or endometrial carcinoma was recorded where material was available.

Statistical methods

Survival time, calculated from time of operation to death due to the tumor, was statistically analyzed using the Kaplan-Meier method. Computations were performed with the BMDP-program 1L (life tables and survival functions). Equality of survival curves is tested with the Mantel-Cox statistic. The significance level is 5%. The same method was utilised for analyzing disease-free

survival, calculated from the time of operation to recurrence of the tumor or death due to the tumor.

Results

Age: The mean age at diagnosis in this series was 49.8 years with an age range from 15 to 79 years (Fig. 3.1).
Stage: Forty-five patients (74%) had stage I disease at the time of operation, 7 patients (11%) had stage II, 6 patients (10%) stage III and 3 patients (5%) stage IV disease (Fig. 3.2).
Pathological features: Fifty-seven (93%) tumors were unilateral, only 2 (3%) were bilateral and in 2 cases this was unknown (Table I).

The neoplasms ranged in size from 1 cm to 33 cm, the mean diameter was 21.8 cm and only 10 tumors had a diameter of 5 cm or less (Fig. 3.3).

Eight tumors (13%) showed spontaneous rupture of the ovarian capsule at the time of operation, 6 belonging to stage I and 2 to higher stages. In 1 neoplasm capsular invasion was detected microscopically.
Histological features: The growth pattern of each tumor was classified as insular, trabecular, microfollicular, macrofollicular, diffuse (for illustrations see chapter 2) and mixed with presence or absence of Call-Exner bodies. Thirty neoplasms (49%) revealed a mixture of various above named patterns, a diffuse pattern being present in 36 (59%) tumors. In 8 neoplasms (13%) granulosa cells were seen lying in spaces resembling lymphatic vessels but it was difficult to be certain whether this signified a true lymphatic invasion.

Nuclear pleomorphism was absent in 46 neoplasms, slight, in 8 tumors, moderate in 5 and marked in 2 tumors. The cells in 46 tumors showed no nuclear hyperchromasia while those in 11 revealed mild, in 3 moderate and in 1 tumor a marked hyperchromasia.

Forty-eight neoplasms showed 0-9 mitoses per 10 H.P.F., 9 revealed 10-29 mitoses per 10 H.P.F. and in 4 tumors 30-55 mitoses

Fig. 3.1

AGE DISTRIBUTION OF PATIENTS WITH GRANULOSA-CELL TUMOR

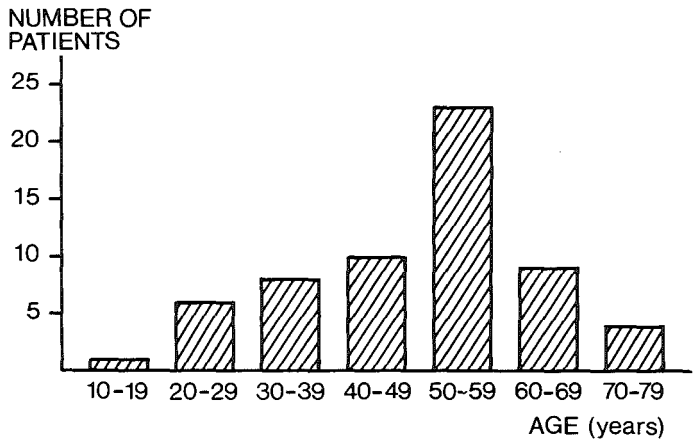


Fig. 3.2 STAGE DISTRIBUTION OF 61 CASES

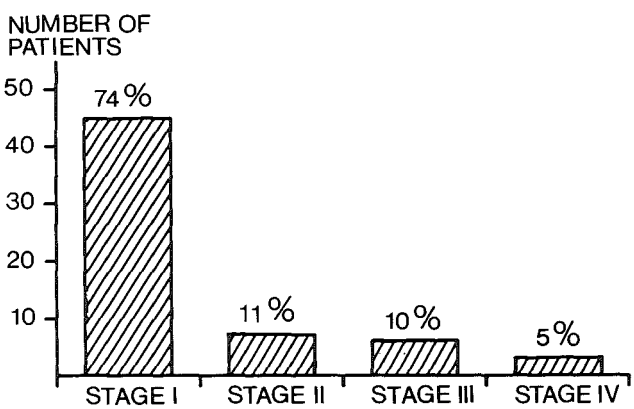
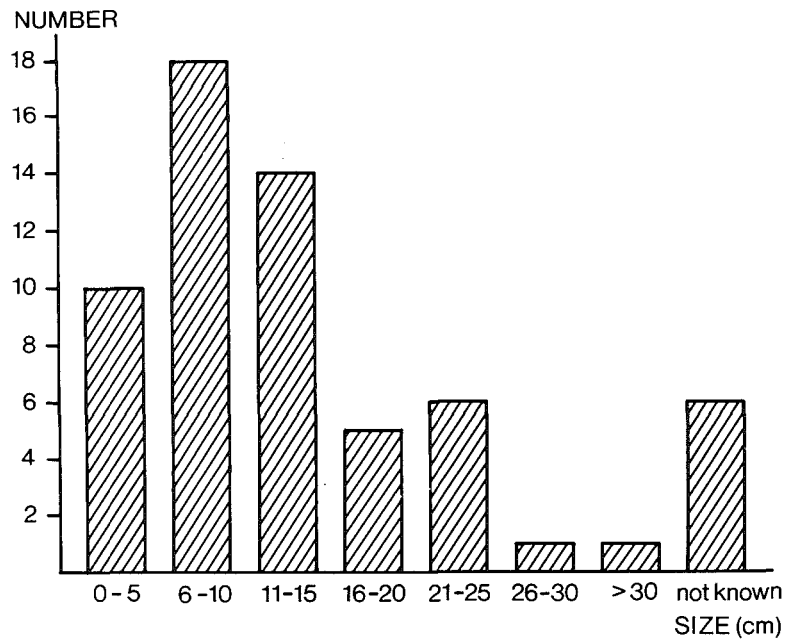


Table I Bilaterality in 61 Granulosa Cell Tumors

	Number of cases	Percent
Unilateral	57	94
Bilateral	2	3
Unknown	2	3

Fig. 3.3

TUMOR SIZE DISTRIBUTION OF 61 GRANULOSA-CELL TUMORS



were present per 10 H.P.F. No significant difference of mitotic rate was found between different areas in one tumor.

Forty-nine tumors showed no necrosis while 9 showed sporadic small areas of necrosis and in 3 tumors a few larger necrotic areas were present.

Associated endometrial changes: In 17 cases (51% of those examined) endometrial hyperplasia was present and in 3 (9%) an endometrial adenocarcinoma was seen. (Table II).

Treatment: The therapeutic regimes employed for patients in the present series varied considerably and are summarized in Table III. Besides, the operative treatment which varied from unilateral oophorectomy (14 patients) to hysterectomy with bilateral salpingo-oophorectomy (37 patients), 13 patients were treated with radiotherapy and 17 had chemotherapy.

Ten patients died from their granulosa cell tumor, 7 of these within 5 years, another 3 patients died from unrelated causes i.e. cardiovascular disease, colon and lung carcinoma.

Table II Endometrial findings in 61 patients with granulosa cell tumor

Endometrium	Number of cases
Hyperplasia	17
Carcinoma	3
Normal	13
Not available	28

Table III Treatment of Patients with Granulosa Cell Tumor

Treatment	Number of patients
Unilateral oophorectomy	9
Unilateral oophorectomy and radiotherapy	1
Unilateral oophorectomy and chemotherapy	3
Unilateral oophorectomy, radiotherapy and chemotherapy	1
Bilateral oophorectomy and radiotherapy	1
Bilateral oophorectomy and chemotherapy	1
Bilateral oophorectomy, radiotherapy and chemotherapy	1
Hysterectomy and unilateral salpingo-oophorectomy	3
Hysterectomy and bilateral salpingo-oophorectomy	27
Hysterectomy bilateral salpingo-oophorectomy and radiotherapy	3
Hysterectomy bilateral salpingo-oophorectomy and chemotherapy	3
Hysterectomy bilateral salpingo-oophorectomy, radiotherapy and chemotherapy	6
Chemotherapy	2

Factors affecting prognosis:

For the whole series the overall 5 year survival is 87% while the 10 year survival is 82% (Fig. 3.4). It was attempted to evaluate to what extent clinical and histopathological findings relate to prognosis by analysing the effect of all the above variables separately on the entire group of patients and on only those with stage I disease.

Age: Patients younger than 40 years did better than those above 40, no patients dying in the former group (13 cases). The difference was nearly statistically significant for all stages combined ($p = 0.07$) (Fig. 3.5).

Stage: Separating stage I disease patients from those in stage II-IV a highly significant difference in survival was noted. In stage I and stage II-IV the 5 year survival is 98% versus 60% respectively and 10 year survival is 95% versus 55% respectively. The relative survival curves differed significantly ($p = 0.0001$) (Fig. 3.6).

Tumor rupture: In stage I disease 1 out of 6 patients with a ruptured tumor died in contrast to none of the 39 patients with unruptured tumor. The former appears to be statistically significant ($p = 0.01$).

Size: Patients with neoplasms larger than 5 cm were at greater risk than those with 5 cm diameter or less. The difference is, however, not statistically significant for all stages combined ($p = 0.28$). In stage I 32 patients had a tumor diameter greater than 5 cm and 10 below 5 cm. In the former group 1 patient died which is not statistically significant.

Growth pattern: Considering the histological growth pattern as a sole prognostic factor it appeared that the presence of a diffuse growth pattern points to a poor prognosis. Thus, the difference in prognosis between tumors with and without a diffuse growth pattern appeared to be statistically significant ($p = 0.0001$). If the clinical staging was taken into account a different result emerged. For this purpose 4 groups of tumors were distinguished:

1. stage I tumors without diffuse growth pattern.
2. stage I tumors with diffuse growth pattern.

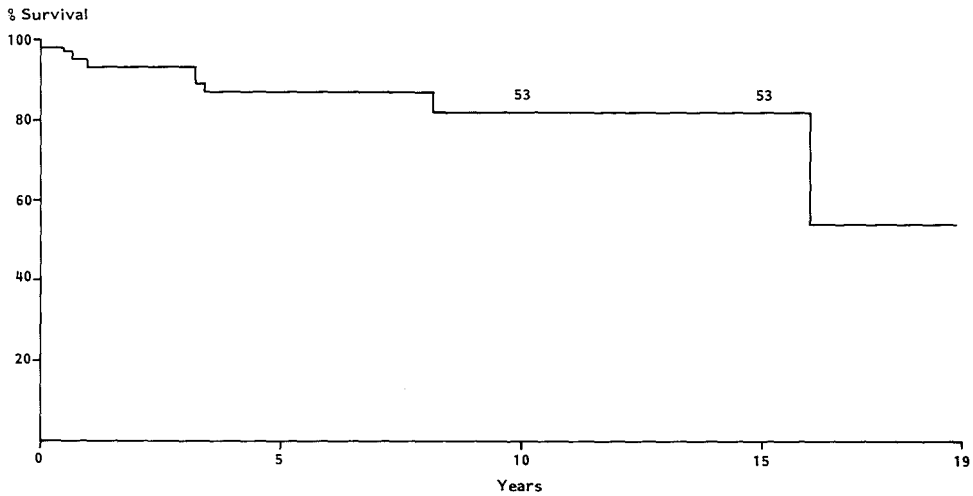


Fig.3-4 Overall survival of 61 patients. Numbers on survival curve denote number of patients alive after 10 and 15 years observation.

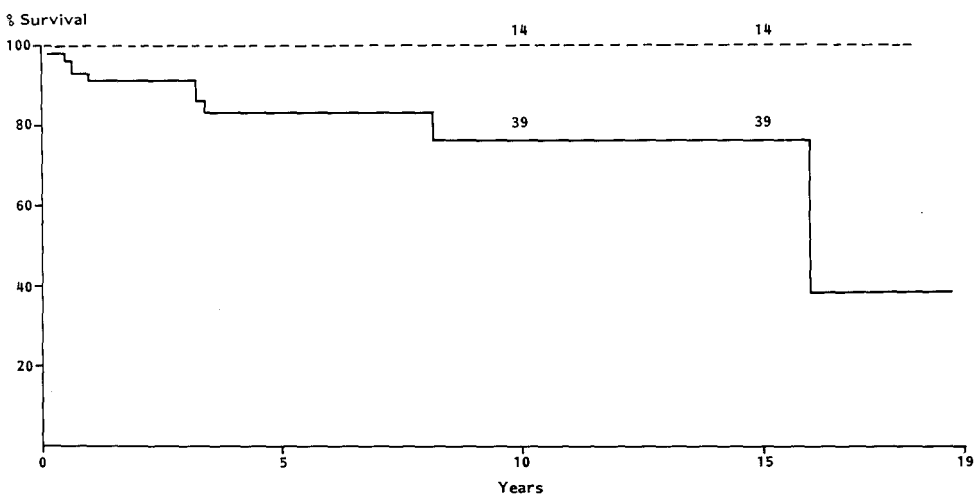


Fig.3-5 Survival of clinical stage I-IV patients below age 40 years (--- n = 14) and above 40 years (——n = 47). Numbers on survival curve denote number of patients alive after 10 and 15 years observation.

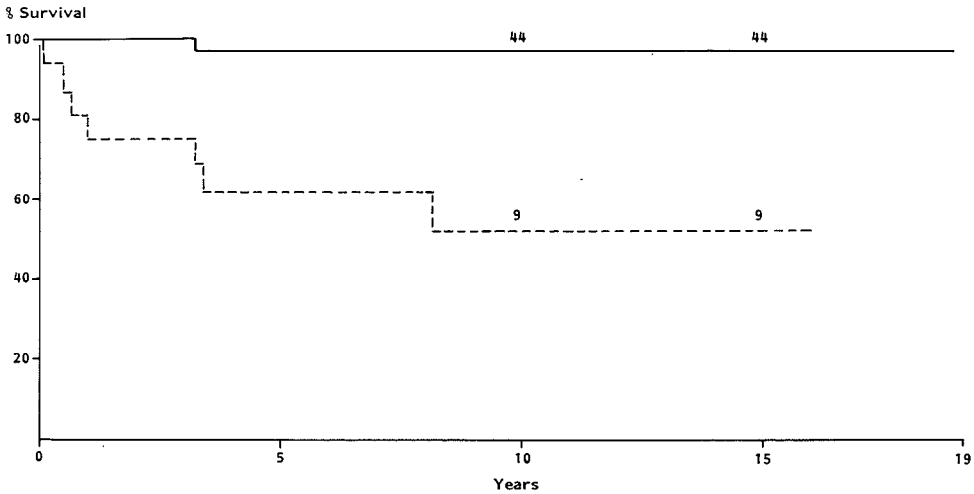


Fig.3-6 Survival of clinical stage I (—— n = 45) and clinical stage II-IV (--- n = 16) patients. Numbers on the survival curve denote number of patients alive after 10 and 15 years observation.

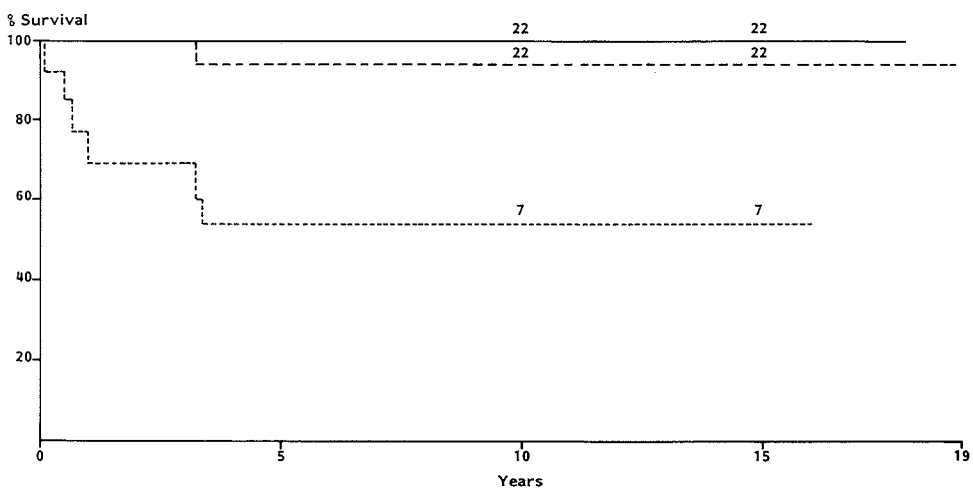


Fig.3-7 Survival for clinical stage I patients with non diffuse histological pattern (——n = 22), stage I with diffuse pattern (--- n = 23) and stage II-IV diffuse pattern (... n = 13). Numbers on survival curves denote number of patients alive in each group after 10 and 15 years observation.

3. stage II-IV tumors with a diffuse growth pattern.
4. stage II-IV tumors without diffuse growth pattern.

The last group consisted of only 3 patients. Fig. 3.7 demontrates the survival curves of group 1, 2 and 3 and it can be deduced from these survival curves that the diffuse growth pattern itself is not associated with a worse prognosis, as the differences in survival are dominated by the staging factor.

Nuclear pleomorphism: The mortality increased with increased nuclear pleomorphism and hyperchromasia for all stages combined and was statistically significant ($p = 0.03$ and $p = 0.04$), respectively (Figs. 3.8 and 3.9).

Mitotic activity: No significant influence of mitotic activity on survival was found in the present series. Both in stage I and higher stage patients the mitotic index did not seem to bear any significant relation to prognosis.

Lymphatic invasion: No significant relationship was detected between what appeared to be lymphatic invasion and prognosis.

Multivariate analysis: In order to evaluate the effect of each factor on survival while taking into account the other prognostic factors, a multivariate analysis (such as Cox-regression) would theoretically be appropriate. However, due to a number of data missing and specially because the mortality rate was relatively low, this model could not be utilized. Nevertheless, after checking the associations between the studied factors, only nuclear pleomorphism and hyperchromasia appeared to be significantly associated (X -test, $p = 0.0001$). Consequently, except for nuclear pleomorphism and hyperchromasia none of the other factors studied will substantially change the prognostic value if other factors are taken into account.

Discussion

Since there is no consensus of opinion in the literature regarding the correlation of different clinical and morphological factors with prognosis in granulosa cell tumors, this investi-

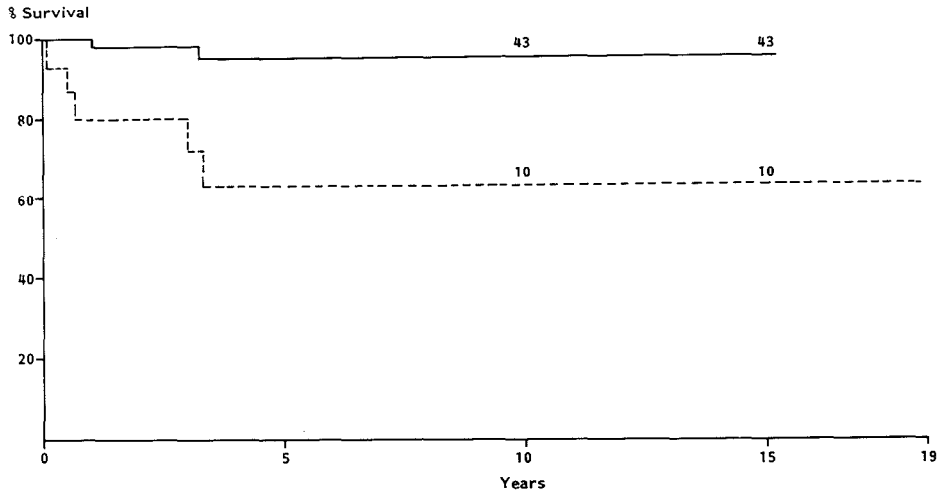


Fig.3-8 Survival of patients (all stages) with nuclear pleomorphism (—— n = 46) and without nuclear pleomorphism (--- n = 15). Numbers on survival curves denote number of patients alive after 10 and 15 years observation.

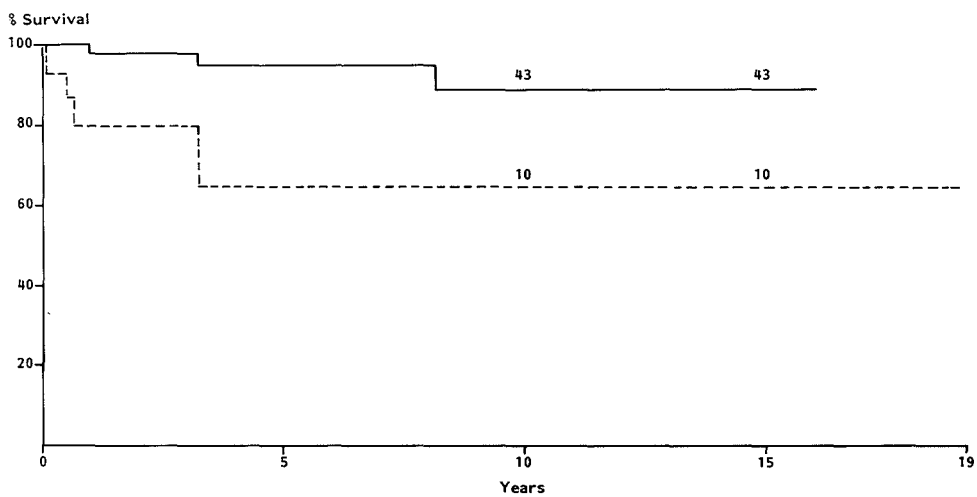


Fig.3-9 Survival of patients (all stages) with nuclear hyperchromasia (—— n = 46) and without nuclear hyperchromasia (--- n = 15). Numbers on survival curve denote number of patients alive after 10 and 15 years observation.

gation was carried out to ascertain this relationship in our series.

The percentage of patients presenting at stage I in this study (74%) is slightly lower than that reported (Stenwig, Hazekamp & Beecham, 1979; Bjorkholm & Silfversward, 1981; Young & Scully, 1984). The 10 year survival of 97% for stage I disease and 52% for higher stages is comparable to the results obtained by the above mentioned investigators. Also the mean age at diagnosis of 49.8 years is similar to the findings of other authors (Fox, Agrawal & Langley, 1975; Evans et al., 1980; Bjorkholm & Petterson, 1980). Age seems to influence prognosis as patients below age 40 do better than patients above 40 (Fig. 3.5). However, this difference is not statistically significant.

The occurrence of bilaterality for granulosa cell tumors in this study (3%)is comparable with the frequency observed in previous studies (Norris & Taylor, 1968; Stenwig, Hazekamp & Beecham, 1979).

The present report and the previous studies are comparable since the average age of patients studied, tumor stage and bilaterality did not vary greatly.

A 100% 10 year survival of patients with tumors 5 cm or less in diameter in contrast to the 81% 10 year survival for neoplasms above 5 cm is also in accordance to the tumor size prognosis relationship reported in the literature (Bjorkholm & Silfversward, 1981; Stenwig, Hazekamp & Beecham, 1979; Fox, Agrawal & Langley, 1975; Sjostedt & Wahlen, 1961). On the other hand no significant difference in survival was observed in stage I patients with tumors smaller and larger than 5 cm diameter. In literature no data established a relation between tumor size and prognosis independent of stage (Young & Scully, 1984).

Rupture of the tumor in stage I disease adversely affected the prognosis as has been previously reported (Bjorkholm and Silfversward, 1981).

In the literature contradictory results were noted on the relationship of histological growth pattern and prognosis. A long follow-up appeared to be necessary before survival data of

patient series with GCT become meaningful (Young & Scully, 1984). In a long term follow-up study Stenwig et al. considered the prognostic value of histological pattern of GCT in relation to the clinical stage. These authors did not observe a significant influence of this latter factor on prognosis. The presence of a diffuse histological growth pattern did not have any prognostic value in this series. It was striking that most patients in high stage had a tumor with diffuse histological pattern.

The degree of nuclear atypia found in this study bearing a relationship to prognosis has also been found by others (Stenwig, Hazekamp & Beecham, 1979; Bjorkholm & Silfversward, 1981). However, Young & Scully in their recent study (1983) of a group of GCT, Sertoli-Leydig cell tumors and thecomas found no evidence of influence of presence of bizarre nuclei on prognosis. They considered these to be probably of degenerative nature.

Mitotic activity in granulosa cell tumors in this series did not have any correlation with prognosis which is in contrast with the results of earlier reports (Fox, Agrawal & Langley, 1975; Stenwig, Hazekamp & Beecham, 1979; Bjorkholm & Silfversward, 1981). Even for higher stage patients we did not find such a correlation.

Norris and Taylor (1968) reported lymphatic invasion in 4 granulosa cell tumors and 3 mixed granulosa theca cell tumors in which there were 2 recurrences (29%). In the present series "lymphatic invasion" did not correlate with prognosis perhaps confirming our suspicion that such observations did not signify true lymphatic invasion, but may be due to contamination.

Well known is the association of granulosa cell tumor with endometrial hyperplasia and adenocarcinoma which was confirmed by us. A 10% incidence of malignancy found in women whose endometrium was studied is consistent with that reported by Fox et al. (1975), but higher than the frequency of 5% reported by Scully (1977).

Although very little could be said concerning the correlation between treatment regimes and prognosis since the type of treatment has been extremely variable in this study, the type of surgical treatment did not show any statistically significant

difference in stage I disease when compared to all stages combined.

In conclusion in our study the following features were indicative of a relatively poor prognosis:

1. Patients older than 40 years at the time of diagnosis
2. FIGO stage at the time of diagnosis
3. Rupture of the tumor in stage I disease
4. Tumor diameter above 5 cm
5. Presence of nuclear pleomorphism and hyperchromasia.

The factors that did not correlate with prognosis were mitotic activity, diffuse histological pattern,lymphatic invasion and type of surgical treatment.

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CHAPTER 4

FLOW CYTOMETRIC ANALYSIS OF NUCLEAR DNA CONTENT IN OVARIAN GRANULOSA CELL TUMORS

Introduction

Granulosa cell tumors which account for less than 2% of all ovarian tumors and less than 10% of malignant ovarian tumors (Scully, 1979) belong to the category of sexcord-stromal tumors. Together with theca cell tumors they constitute the most common functioning ovarian neoplasms. The malignant potential of these tumors is debated (Fox, Agrawal & Langley, 1975; Norris & Taylor, 1968). Generally all granulosa cell tumors are moderately malignant neoplasms which may have a prolonged course with a tendency to late recurrence. Till now no unequivocal clinical or histological prognostic factors have been identified which can predict the degree of malignancy of granulosa cell tumors (Bjorkholm, & Petterson, 1980; Fox, Agrawal & Langley, 1975).

An association between aneuploidy and aggressive biological behaviour has been demonstrated in epithelial ovarian cancer (Friedlander et al., 1983; 1984a; 1984b; Erhardt, Auer & Bjorkholm, 1984; Volm, Bruggemann & Gunther, 1985). A similar relationship has been shown in a variety of other malignancies (Gustafson, Tribukait & Esposti, 1982; Wolley et al., 1982; Auer et al., 1984; Hedley et al., 1984; Cornelisse, Van de Velde & Caspers, 1987).

In the present paper we report the results of a retrospective study investigating the prognostic value of DNA ploidy in granulosa cell tumors of the ovary. The study comprised 50 patients with granulosa cell tumor whose course was known with follow-up periods ranging from 4 months to 19 years.

Materials and Methods

Since 1966 105 tumors, considered to be granulosa and granulosa theca cell tumors have been registered with the Netherlands

Committee for Ovarian Tumors. Follow-up information could be obtained in only 51 of these patients. Ten more cases were available which had been referred for treatment from the regional hospitals to the Rotterdam Radiotherapy Institute. Patients ranged in age from 15 to 79 years with a mean age at diagnosis of 49.8 years.

Paraffin embedded tissue blocks were available in 50 cases which form the basis of this study. In 49 material was available from the primary tumor only; in 1 case material from the primary tumor as well as from the metastasis was available. Follow-up of these patients ranged from 4 months to 19 years with a median follow-up of 3 years and 9 months. All tumors had been staged according to the recommendation of International Federation of Gynecology and Obstetrics (FIGO). Additional frozen samples of fresh tumor tissue stored at -70°C were available from 6 cases.

Immunohistochemistry

Immunohistochemistry was performed to demonstrate the presence of intermediate filament proteins vimentin and cytokeratin.

Paraffin sections (5 µm thick) were deparaffinized in xylene and then placed in 100% ethanol. The endogenous peroxidase activity was blocked by immersing the sections in a solution of methanol containing 3% hydrogen peroxide for 30 minutes. The sections were then subjected to a proteolytic treatment using 1% protease (Sigma) in phosphate buffered saline (PBS) for 25 minutes at 37°C. Non-specific background staining was reduced by treating the sections with an 1 in 5 dilution of 20% normal (non-immune) swine serum for 15 minutes. Subsequently sections were incubated with rabbit antisera specific for vimentin or keratin (Eurodiagnostics, Holland) and finally incubated with peroxidase conjugated swine anti-rabbit serum IgG (Dako, Denmark) in 1 in 20 dilution for 30 minutes. Following each incubation except after the preincubation with normal swine serum sections were washed thoroughly with PBS. Antibody localisation was determined on the basis of peroxidase activity effected by 10 minutes incubation of

the sections with a TRIS buffered saline solution (0.05 M, pH 7.4) containing 50 mg % 3,3 diamino-benzidine-tetrahydrochloride (Fluka, FRG) and 0.03% hydrogen peroxide. Slides were then washed with water, counterstained with hematoxylin and mounted in Malinol.

Flow Cytometry (FCM)

Nuclear DNA content of paraffin embedded tumor tissue was analysed by FCM using the method of Hedley et al. (1983). In all cases the paraffin blocks used for DNA measurements were checked for the presence of sufficient tumor tissue by examining the hematoxylin-azofloxin stained sections. In the majority of the cases the percentage of tumor cells was approximately 80%, the lower limit being 30%. Thirty-five µm sections were cut from one to three paraffin blocks of tumor and an adjacent 5 µm section for hematoxylin-azofloxin staining. The sections were placed in glass centrifuge tubes and then rehydrated in 100, 96, 70 and 50% ethanol for 10 minutes each at room temperature after which the tissue was washed in two changes of distilled water and resuspended in 1 ml of 0.5% pepsin in 0.9% NaCl (pH 1.5). The tubes were placed in a water bath at 37°C for 30 minutes with intermittent vortex mixing. The cell suspension was then centrifuged, supernatant fluid removed and the pellet suspended in 1 µg per ml 4'6'-diamidino-2-phenylindole dihydrochloride (DAPI) for 45 minutes at room temperature and then filtered through nylon gauze. Suspensions of single cell nuclei were prepared from frozen tissue blocks with the detergent-trypsin procedure of Vindeløv (1983) and stained with propidium iodide (PI). Rainbow trout red blood cells were added to the suspensions of isolated nuclei as an internal ploidy standard.

The stained samples were measured on an ICP 22 flow cytometer (Ortho, Westwood, Mass. U.S.A.). For excitation of DAPI fluorescence a 365 UGI filter and 400 nm chromatic beam splitter was used. Emission was measured using a 435 GG barrier filter. For excitation of propidium iodide fluorescence, LP 515 and SP 560 filters were used in combination with a 560 nm chromatic beam

splitter. Emission was measured using a LP 590 barrier filter. Filtered demineralised water was used as a sheath fluid.

Classification of ploidy abnormalities

The ploidy of the tumor was expressed by the DNA index (DI) being the ratio between the modal channel number of the G0,1 peak of the tumor cell population and that of the G0,1 peak representing the non-neoplastic cell population present in tumor samples. When the DNA profile showed two different G0,1 peaks the one on the left was considered to represent normal diploid cells. Tumors with a DI of 1.0 were classified as diploid and those with two distinct G0,1 populations which per definition have a DI more than 1.0, were classified as aneuploid.

Statistical analysis

The survival curves were calculated according to the Kaplan Meier method (1958) and differences between groups were tested by the Logrank test (Mantel, 1966).

Results

Histology and Immunohistochemistry

Cells of all but 3 tumors constantly expressed vimentin. The 3 vimentin negative tumors were positive for cytokeratin which is not consistent with stromal origin. We rereviewed the histological sections. Forty seven of the fifty tumors showed the typical histological appearances of granulosa cell tumors while the 3 vimentin negative, cytokeratin positive neoplasms focally had solid areas with included spaces resembling Call-Exner bodies but lacked the typical coffee bean nuclei. On the basis of these morphological features and immunohistochemistry these 3 tumors were considered to be undifferentiated carcinomas.

Flow cytometry

DNA profiles with only a single G0,1 peak were obtained for 38 archival deparaffinized tumor specimens, whereas in 5 cases two distinct G0,1 populations were found. The latter were classi-

fied as aneuploid, considering the peak with the highest modal fluorescence value as the tumor stemline. The DNA indices of the five aneuploid tumors were 1.16, 1.20, 1.49, 1.81 and 1.84 respectively. The 38 cases with a single G0,1 peak showed a considerable variation of the coefficient of variation (CV) which ranged from 2.4% to 13.7%. A positive correlation was found between the CV and the age of the blocks (rank correlation coefficient of Spearman $p = 0.01$). In contrast low CV's were found for the G0.1 peaks from 6 frozen specimens (1.7% to 3.0%). With the range of CV's found for the archival specimens it is not possible to discriminate between tumors with a true diploid DNA content and those with minor ploidy abnormalities. Therefore all DNA profiles from deparaffinized archival specimens showing only a single G0,1 peak have been classified as (near-)diploid. In Fig. 4.1 the (near-)diploid DNA profile (CV = 3.4%) of a granulosa cell tumor with a microfollicular pattern and Call-Exner bodies is shown. Fig. 4.2 shows a (near-)diploid DNA profile of granulosa cell tumor (CV = 9.0%) with a diffuse histological pattern with coffee bean nuclei. DNA profiles of a primary granulosa cell tumor with a diffuse arrangement of granulosa cells (a) and its metastasis (b) both showing (near-)diploid stemlines are presented in Fig. 4.3. Fig. 4.4 shows the aneuploid DNA profile and histology of one of the three vimentin negative keratin positive tumors lacking coffee bean nuclei.

In 7 cases the DNA profile showed a debris continuum only and could not be evaluated. Table 1 summarizes the tumor DNA ploidy results of paraffin embedded tumor tissue from 43 patients.

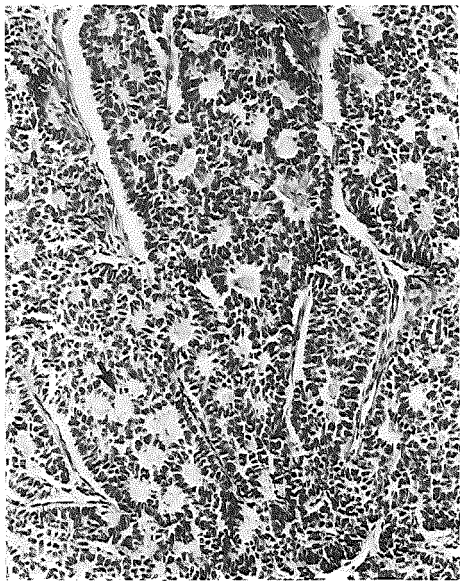

Fig.4-1. Near-diploid DNA profile with low CV (3.4%) of a granulosa cell tumor showing microfollicular pattern with Call-Exner bodies (arrow).

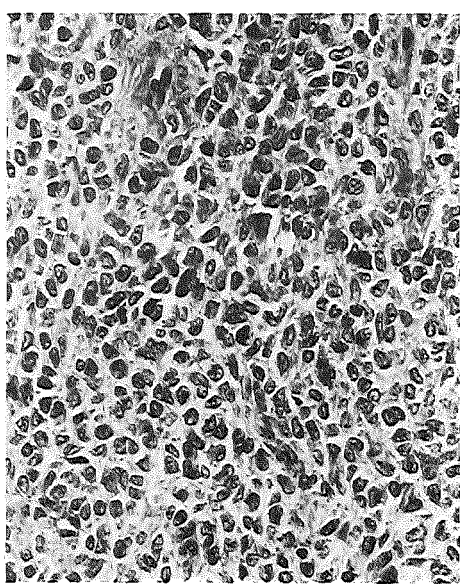

Fig.4-2. Near-diploid DNA profile with high CV (9.0%) of a granulosa cell tumor and a diffuse pattern showing coffee-bean nuclei.

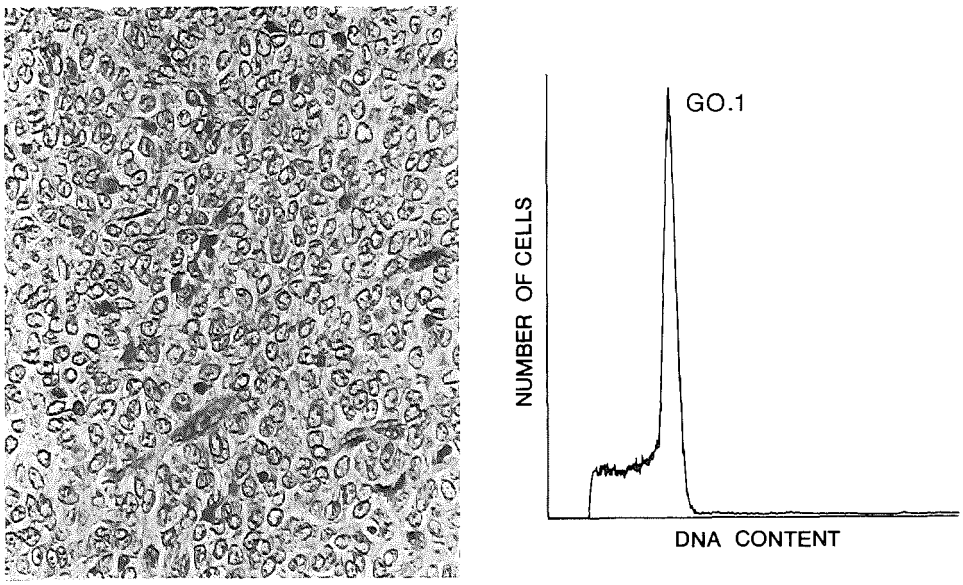

Fig.4-3a. Near-diploid DNA profile of a diffuse type of granulosa cell tumor (CV = 4.3%).

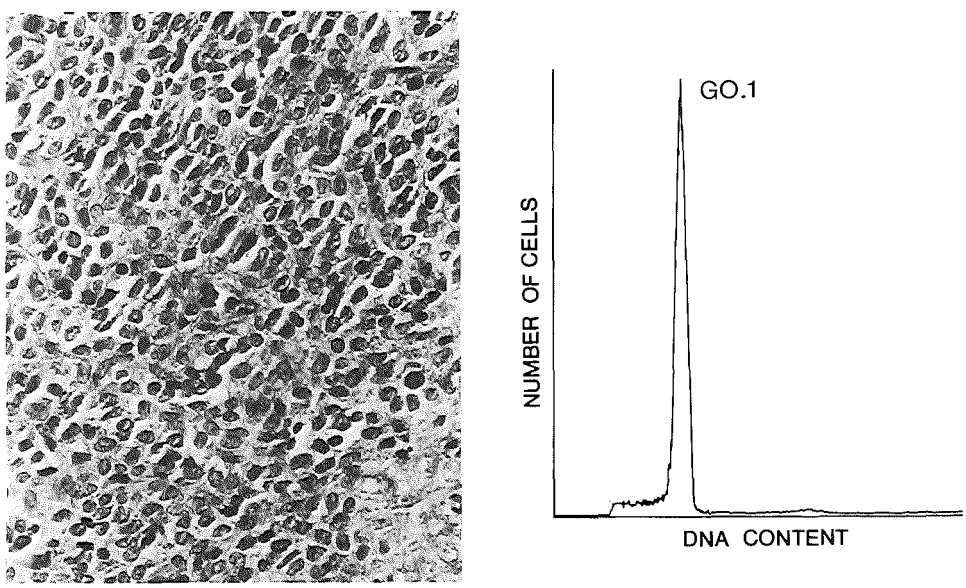

Fig.4-3b. Near-diploid DNA profile of metastasis from tumor in Fig.3a (CV = 5.3%).

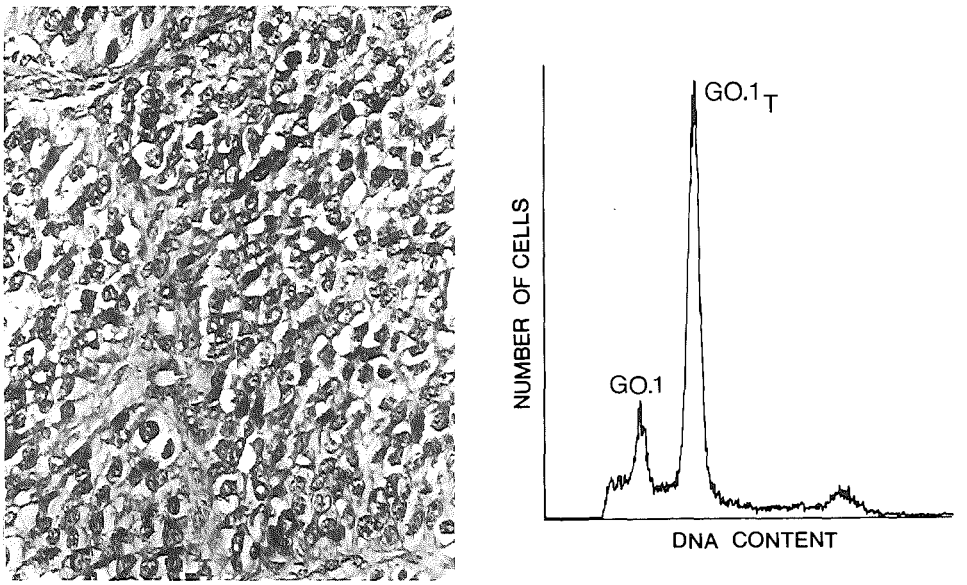

Fig.4-4. Aneuploid DNA profile of an undifferentiated carcinoma - lacking coffee-bean nuclei. $G0,1_T$ = aneuploid G0,1 fraction.

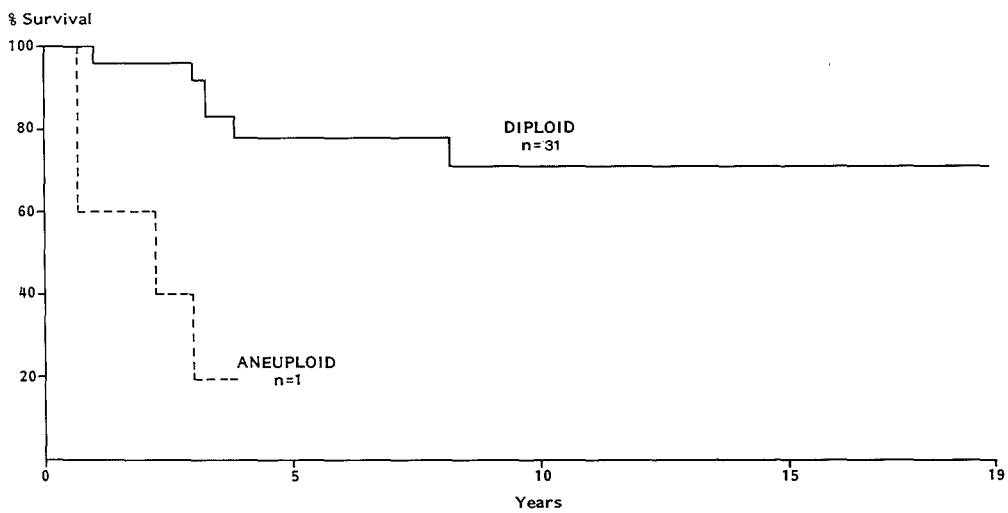

Fig.4-5. Survival by ploidy. There is significant difference between survival of patients with diploid and aneuploid tumors. (P = 0.0002). Aneuploid tumors include 3 undifferentiated carcinomas. n = patient number surviving in each group.

Table I

Ploidy distribution in relation to FIGO stages

Stage	(Near-)diploid (38 cases)	aneuploid (5 cases)
I	24 (63%)	1 (20%)
II	6 (16%)	1 (20%)
III	4 (10.5%)	1 (20%)
IV	4 (10.5%)	2 (40%)
	DNA index 1.0	DNA index 1.16-1.84

Ploidy survival

The results of the survival analysis show (Fig. 4.5) that all five patients with DNA aneuploid tumors had a significantly shorter survival time than those with a (near-)diploid tumor ($p = 0.0002$). From thirty eight patients with (near-)diploid tumors thirty-one are still alive compared to one out of five with aneuploid tumors. A high CV was not related to survival in the (near-)diploid group. Seven patients who died in the (near-) diploid group had a granulosa cell tumor in different FIGO stages; three had stage I, two stage II and two stage IV disease. Two of the patients with stage I granulosa cell tumor died due to other causes (lung cancer and heart disease). The other five patients died due to recurrence of granulosa cell tumor. Of five patients with aneuploid tumors one had stage I disease, one stage IIb, one stage III and two stage IV disease. Four patients from this group died of their malignant disease. One patient remaining with stage I disease was lost to follow-up after 4 years.

Discussion

In this study 38 of the 43 (88%) tumors primarily by light-microscopy diagnosed as granulosa cell tumors were (near-)diploid while 5 (12%) were DNA aneuploid. This is in contrast to the

results reported for ovarian carcinomas (Friedlander et al., 1983; Volm, Bruggemann & Gunther, 1985; Rodenburg et al., 1987). Results of Friedlander et al. showed 76% (38 of 50 cases) aneuploidy versus 24% (12 of 50 cases) diploidy and results of Rodenburg et al. 61% (45 of 74 cases) aneuploidy versus 39% (29 of 74 cases) (near-)diploid. Volm et al. in their series reported 71% aneuploidy versus 29% diploidy. In cancers of various organs diploidy is associated with a significantly better prognosis as compared to DNA aneuploid tumors. Friedlander et al. (1983), Volm et al. (1985) and Rodenburg et al. (1987) have shown that DNA ploidy is an important prognostic indicator in ovarian epithelial cancer.

It is known that granulosa cell tumors of the ovary have a low malignant potential with a favourable prognosis and a tendency to late recurrence. Thus the predominance of (near-) diploid stemlines seems to be compatible with and perhaps related to the known relatively favourable prognosis of these tumors. Patients with (near-)diploid tumors in this series showed a much better survival than patients with DNA aneuploid tumors. Thirty one out of 38 patients with near-diploid tumor are still alive as compared to 1 out of 4 with aneuploid tumors. Two of the 7 patients in the near-diploid group died of other causes (lung cancer and heart disease) and the remaining 5 died due to recurrence of granulosa cell tumor. In the DNA aneuploid group 4 out of 5 patients died of their malignant disease. Three patients showing high aneuploidy (DI 1.49-1.84) died within 1, 2 and 3 years respectively after operation. These three patients were also in higher stage (IIb, III, IV) which is comparable with previous observations on epithelial ovarian cancer showing that diploid tumors tend to be of low stage while aneuploid ones are of high stage (Friedlander et al., 1983).

These 3 tumors were vimentin negative and cytokeratin positive. They also lacked the typical histological features of granulosa cell tumor. As stated earlier, these tumors were considered to be undifferentiated carcinomas, they were aneuploid and had a bad prognosis. The other 2 aneuploid tumors, however, had lower DNA indices (1.16-1.20). One of these patients died

within 8 months with intestinal and pulmonary metastases (stage IV) the other patient was lost to follow-up after 4 years (stage I).

In this series tumors classified as (near-)diploid had a large range of CV's, those with a high CV indicating perhaps the possibility of low aneuploidy (two G0,1 peaks merged in 1 leading to a high CV). However, no relation was found between a high CV and survival. Another possible explanation of the high CV's may possibly be found in the age of the paraffin blocks, some of the blocks being as old as 19 years. Considering that the paraffin embedded material was obtained from different laboratories, the tumor material fixation may have been substandard in a number of cases e.g. by using unbuffered formalin which could lead to DNA loss by acid hydrolysis.

In conclusion the results of our study of ovarian granulosa cell tumors indicate that a) the majority of these neoplasms is diploid or has minor ploidy abnormalities not detectable by flow cytometry on deparaffinized tissue sections; b) an undifferentiated carcinoma should be considered in the differential diagnosis of tumors with a high DNA index. Moreover, the results suggest that the presence of an aneuploid stemline is associated with a worse prognosis.

Therefore flow cytometry may be of potential value for the differential diagnosis and prognostic grading of granulosa cell tumors.

To substantiate these conclusions a prospective flow cytometric study on fresh samples of granulosa cell tumors seems desirable in order to circumvent the problems of the use of archival paraffin embedded material.

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CHAPTER 5

IMMUNOHISTOCHEMISTRY OF OVARIAN GRANULOSA CELL TUMORS: THE VALUE OF TISSUE SPECIFIC PROTEINS AND TUMOR MARKERS

Introduction

Ovarian neoplasms comprise a group of histomorphologically and functionally different tumor types. Most of these can be diagnosed by routine examination of adequate numbers of sections stained by hematoxylin eosin with the occasional help of special stains, (Scully, 1984). However, it may be difficult to differentiate between tumors lacking clear histological differentiation; i.e., the differentiation between poorly differentiated sexcord-stromal tumors from undifferentiated carcinomas or carcinoid tumors. Immunohistochemical techniques have therefore been used recently in the diagnosis and study of ovarian neoplasms (Kurman, Ganjei & Nadji, 1984).

So far only a limited number of studies report on the diagnostic use of intermediate filament proteins in ovarian tumors (Miettinen, Lehto & Virtanen, 1983; Bonazzi del Poggetto et al., 1983; Kurman et al., 1984; Miettinen et al., 1985). At present the practical contribution of tumor markers has been greatest in germ cell tumors. A few sexcord-stromal tumors (Sertoli-Leydig cell tumors) have been associated with elevated levels of alpha-fetoprotein in the serum (Benfield, Tapper-Jones & Stont, 1982; Young, Perez-Atayde & Scully 1984; Chumas et al., 1984; Mann et al., 1986; Tetu, Ordonez & Silva, 1986; Chadha, Honnebier & Schaberg, 1987). In two cases this antigen was localised in areas with hepatocyte differentiation within a retiform Sertoli-Leydig cell tumor with heterologous elements (Young, Perez-Atayde & Scully, 1984; Chadha, Honnebier & Schaberg, 1987).

The aim of the present study was to evaluate the usefulness of immunohistochemical demonstration of intermediate filament proteins and epithelial membrane antigen in a group of ovarian neoplasms primarily diagnosed as granulosa cell tumors. In addition the presence of a number of tumor markers and oncofetal

antigens was investigated.

Materials

From the files of the Dutch Ovarian Tumor Committee 31 granulosa theca cell tumors were retrieved. Fourteen cases primarily diagnosed as granulosa cell tumors and referred from regional hospitals to the Rotterdam Radiotherapy Institute were also included in the study. Formalin fixed and routinely paraffin embedded tissue blocks were available from the above cases. In addition frozen sections from 3 granulosa cell tumors were studied. The histological diagnosis was based on sections stained with haematoxylin azofloxin, using standard criteria (Fox & Langley, 1976; Scully, 1979). Based on an orientating pilot study and the literature we decided to study the following antigens and markers: vimentin, keratin, epithelial membrane antigen (EMA), carcinoembryonic antigen (CEA) and alpha-fetoprotein (AFP). A Grimelius stain was also performed on all tumors.

Methods

Antibodies

The mouse monoclonal antibodies and the rabbit antisera used in the present study are listed in Table I.

To detect the binding of mouse monoclonal antibodies on tissue sections we applied the indirect conjugated method using a rabbit-anti mouse antiserum conjugated to horse-radish peroxidase (RAM-PO) purchased from DAKO, Denmark. The binding of rabbit anti-bodies was detected by a horse-radish peroxidase conjugated swine anti-rabbit antiserum (SWAR-PO) purchased from DAKO, Denmark.

Immunohistochemistry

Paraffin sections of 5μ thickness were deparaffinized in xylene and absolute ethanol. The endogenous peroxidase activity was blocked by incubation in methanol containing 3% hydrogen

Table I

Commercial source of monoclonal and polyclonal antibodies used.

Antibodies	Specific for	Commercial source	Dilution
rabbit anti vimentin	vimentin	Eurodiagnostics	1/50
rabbit anti keratin	keratin	Eurodiagnostics	1/50
mouse anti EMA	epithelial membrane antigen	DAKO	1/10
rabbit anti CEA	carcinoembryonic antigen	DAKO*	1/250
mouse anti AFP	alpha-1-feto-protein	Unipath	1/100
rabbit anti HCG	chorionicgonado-tropin (β-chain)	DAKO	1/50
rabbit anti HPLAP	human placental alkaline phosphatase	DAKO	1/200
rabbit anti HPL	human placental lactogen	DAKO	1/400

* after absorption with liver, spleen and stomach powder.

peroxide for 30 minutes at room temperature. For detection of keratin and vimentin sections were subsequently subjected to proteolytic treatment using 0.1% protease (Sigma) in phosphate buffered saline (PBS) for 25 minutes at 37°C. Before the application of primary rabbit antisera reduction of nonspecific background staining was achieved by incubation of the sections with 20% nonimmune swine serum (NSS) diluted in PBS for 15 minutes. The sections were then overlaid with the appropriate dilutions of the primary antibodies for 1 hour at room temperature (see Table I), rinsed for 10 minutes with PBS, incubated for another 30 minutes with the second (conjugated) antibody and rinsed again in PBS for 10 minutes. The RAM-PO was diluted 1:100 in PBS containing 5% nonimmune human serum (NHS) and 5% nonimmune rabbit serum (NRS). The SWAR-PO was diluted 1:100 in PBS containing 5% NHS and 5% NSS. Antibody localisation was visualised by incubation of sections with a TRIS-buffered saline solution (0.05 M, pH 7.4) containing 50 mg % 3,3 diamino benzidine-tetrahydrochloride (Fluka, FRG) and 0.03% hydrogen peroxide. Slides were then washed with running tapwater, counterstained with haematoxylin and mounted in Malinol.

Immunohistochemistry was also performed on snap frozen tissues from 3 granulosa cell tumors. Cryostat sections of 5µ thickness were airdried thoroughly for at least 30 minutes at room temperature and fixed in acetone for 10 minutes. The remaining procedure was identical to that described for paraffin sections. Positive controls of human chorionic gonadotrophin (HCG), human placental lactogen (HPL), human placental alkaline phosphatase (HPLAP) and EMA included sections of placental tissue. Positive controls for AFP and CEA were sections of a hepatocellular carcinoma and a colon carcinoma, respectively. Negative controls omitting the primary antibody were also employed.

Results

Histological findings

Thirty-nine of the 45 neoplasms had the histological appearance characteristic of well-differentiated granulosa cell tumors. These are therefore designated as typical. Twenty-five of these neoplasms were pure granulosa cell tumors with in fourteen a minor theca cell component. The granulosa cells had scanty cytoplasm and oval or angular nuclei with a longitudinal groove, often containing a single nucleolus. The tumor cells were closely but haphazardly arranged in a dense stroma. A variety of histological patterns was observed, i.e. microfollicular, insular, trabecular and diffuse. The microfollicular pattern was characterised by Call-Exner bodies which are small cavities containing eosinophilic material. The diffuse pattern was most frequently observed in our series.

The remaining 6 neoplasms were poorly differentiated; i.e. these were composed of solid areas consisting of epithelial-like cells lacking the coffee-bean nuclei and focally included spaces resembling Call-Exner bodies, and are further designated as atypical.

Immunohistochemical findings

The data are presented in Table II.

Vimentin: All the typical granulosa cell tumors were distinctly positive for vimentin (Fig. 5.1). Vimentin positivity was also observed in the 3 frozen neoplasms which had the typical morphological features of a granulosa cell tumor. On the other hand the six tumors with atypical histological features were essentially negative for vimentin, although in 3 of the 6 widely scattered vimentin positive tumor cells were found. Vimentin staining was strong in the vascular endothelial cells and stromal elements (Fig. 5.2).

Keratin: All the typical granulosa cell tumors and the 3 frozen tumors examined by frozen section technique were keratin negative. The 6 atypical neoplasms were keratin positive, the

Table II

Pat. no.	Age (yrs)	Tumor diam.	Hist.	Vim.	Ker.	EMA	CEA	AFP	Grim.	Patient status
1	37	?	T	+	-	-	-	-	-	A 132 mo
2	47	7.5	T	+	-	-	-	-	-	Lfu 31 mo
3	58	3.5	T	++	-	-	-	-	-	Lfu 4 mo
4	58	?	T	+	-	-	-	-	-	A 132 mo
5	50	20	T	+	-	-	-	-	-	Lfu 14 mo
6	39	5	T	+	-	-	-	-	-	A 132 mo
7	53	12	T	++	-	-	-	-	-	A 132 mo
8	47	3.5	T	+	-	-	-	-	-	A 100 mo
9	44	15	T	+	-	-	-	-	-	Lfu 24 mo
10	39	11	T	+	-	-	-	-	-	A 96 mo
11	44	28	T	+	-	-	-	-	-	A 96 mo
12	20	5	T	+	-	-	-	-	-	A 72 mo
13	51	10	T	+	-	-	-	-	-	A 60 mo
14	53	5.5	T	+	-	-	-	-	-	A 60 mo
15	75	4	T	+	-	-	-	-	-	D 39 mo ht dis
16	32	6	T	+	-	-	-	-	-	A 29 mo
17	52	8	T	+	-	-	-	-	-	A 228 mo
18	61	7	T	+	-	-	-	-	-	A 48 mo
19	61	24	T	+	-	-	-	-	-	A 32 mo
20	27	7.5	T	+	-	-	-	-	-	A 45 mo
21	66	8	T	+	-	-	-	-	-	A 135 mo
22	46	12	T	+	-	-	-	-	-	A 72 mo
23	37	16	T	++	-	-	-	-	-	A 144 mo
24	68	?	T	++	-	-	-	-	-	D 12 mo rec
25	58	10	T	++	-	-	-	-	-	A 85 mo
26	62	10	T	+	-	-	-	-	-	D 39 mo rec
27	51	9	T	+	-	-	-	-	-	D 46 mo lung ca
28	54	22	T	+	-	-	-	-	-	D 98 mo rec

Pat. no.	Age (yrs)	Tumor diam.	Hist.	Vim.	Ker.	EMA	CEA	AFP	Grim.	Patient status	
29	56	?	T	+	-	-	-	-	-	A	60 mo
30	73	?	Atyp	-	+	+++	+	-	-	D	8 mo rec
31	41	?	Atyp	-	+	+++	-	-	-	D	10 mo rec
32	69	?	Atyp	-	+	+++	+	-	-	D	14 mo rec
33	74	?	Atyp	-	+	++	-	-	-	D	27 mo rec
34	53	?	Atyp	-	+	++	-	-	-	D	39 mo rec
35	41	14.5	Atyp	-	++	++	-	-	-	D	50 mo rec
36	70	8	T	+	-	-	-	-	-	A	228 mo
37	42	4	T	++	-	-	-	-	-	A	48 mo
38	48	9	T	++	-	-	-	-	-	A	24 mo
39	46	7	T	+	-	-	-	-	-	A	11 mo
40	43	30	T	++	-	-	-	-	-	A	14 mo
41	24	14	T	+	-	-	-	-	-	A	14 mo
42	54	3	T	+	-	-	-	-	-	A	84 mo
43	47	16	T	+	-	-	-	-	-	A	12 mo
44	47	4.5	T	+	-	-	-	-	-	A	15 mo
45	60	3	T	+	-	-	-	-	-	A	8 mo

Vim = vimentin; Ker = keratin; EMA = epithelial membrane antigen; CEA = carcinoembryonic antigen; AFP = alpha-fetoprotein; Grim = grimelius staining
T = typical; Atyp = atypical
A = alive; D = died; mo = months; Lfu = lost to follow-up; ht dis = heart disease; rec = recurrence

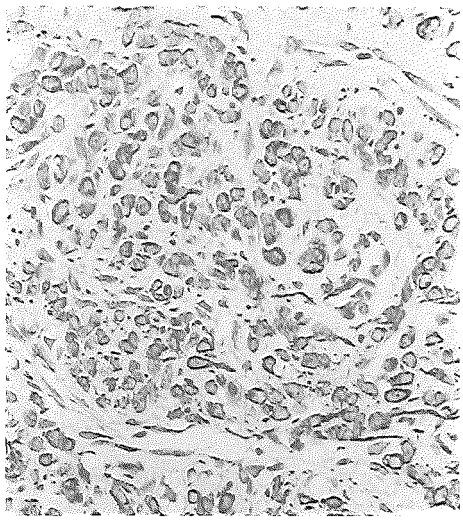

Fig.5-1
A granulosa cell tumor with a diffuse pattern showing vimentin positivity in tumor cells. (Immunoperoxidase Vimentin x380)

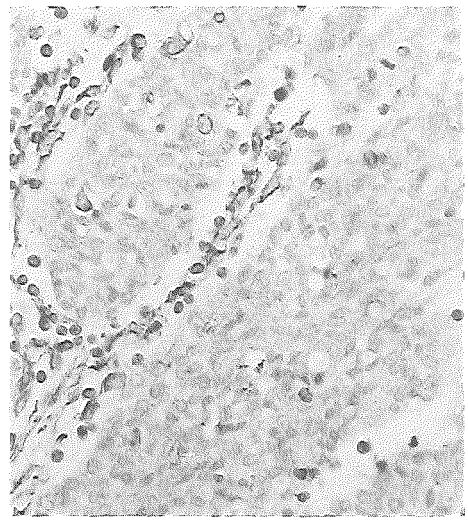

Fig.5-2
An undifferentiated ovarian carcinoma showing vimentin negative tumor cells. Vimentin is only positive in the vascular and stromal septal cells. (Immunoperoxidase Vimentin x380)

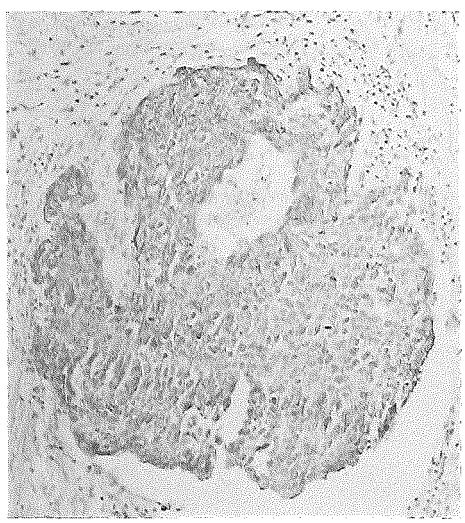

Fig.5-3
An ovarian carcinoma with solid epithelial areas originally diagnosed as granulosa cell tumor showing moderate positivity of tumor cells with keratin. (Immunoperoxidase Keratin x150)

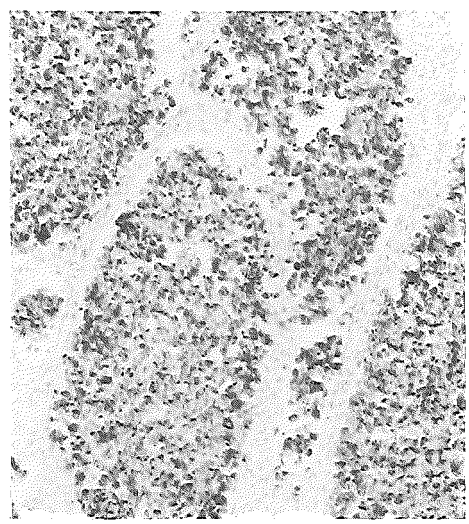

Fig.5-4
An ovarian carcinoma originally diagnosed as granulosa cell tumor showing intense positivity of tumor cells with epithelial membrane antigen. (Immunoperoxidase EMA x150)

intensity of staining being mild to moderate in 10-50% of the tumor cells (Fig. 5.3).

EMA: All the tumors showing typical morphological features of granulosa cell tumor were negative for EMA. The six atypical tumors revealed - in the majority of tumor cells - a distinct and intense positivity for EMA (Fig. 5.4).

CEA: Only two of the 6 tumors classified as atypical were CEA positive. None of the typical granulosa cell tumors were CEA positive.

AFP: None of the typical as well as atypical tumors tested for AFP were positive.

The 6 granulosa cell tumors tested for HCG, HPLAP and HPL as a part of the orientating pilot study showed negative results.

Grimelius: Staining performed on all the typical and atypical tumors was negative.

Survival: The survival of these 45 patients varied from 4 months to 19 years with an average survival of 9 years and 8 months. Survival time of the 6 patients with atypical tumors was 8, 10, 14, 27, 39 and 50 months (see Table II).

Discussion

In general keratin and EMA are well established epithelial markers while vimentin is considered to be a good indicator of a tissue of mesenchymal origin.

The observation that 39 neoplasms with typical morphological features of a granulosa cell tumor were vimentin positive is consistent with the presumed mesenchymal origin of these sexcord-stromal tumors. Moreover, these 39 neoplasms were found to be keratin negative. These results are in accordance with those of Miettinen et al. (1985). In addition we found that all typical granulosa cell tumors were EMA negative.

On the other hand the 6 tumors with atypical histological features, originally diagnosed as consistent with poorly differentiated malignant granulosa cell tumors, were vimentin negative. However, 3 of these neoplasms had scattered vimentin-positive cells as previously observed in some ovarian carcinomas

(Miettinen, Lehto & Virtanen, 1983). All these 6 atypical neoplasms, in addition, were mildly to moderately positive for keratin. Moreover, they had an intense positivity with the anti-EMA antibody. These results suggest that these atypical tumors should be better classified as undifferentiated ovarian carcinomas.

In our series EMA appears to be next to vimentin the marker of choice to discriminate between poorly differentiated ovarian carcinoma and poorly differentiated sexcord-stromal tumors. Similarly other authors have suggested that in comparison to carcinoembryonic antigen and keratin, EMA was the most sensitive marker of epithelial differentiation in formalin fixed tissue (Sloane, Hughes & Ormerod, 1983).

All 45 tumors tested for AFP were negative in contrast to the 8 cases of Sertoli-Leydig cell tumors (androblastomas) described in the literature with evidence of AFP production (Benfield, Tapper-Jones & Stont, 1982; Young, Perez-Atayde & Scully, 1984; Chumas et al., 1984; Mann et al., 1986; Tetu, Ordonez & Silva, 1986; Chadha, Honnebier & Schaberg, 1987) indicating that out of the group of sexcord-stromal tumors androblastomas are more apt to produce AFP.

Because a carcinoid tumor should be considered in the differential diagnosis of a granulosa cell tumor our negative results with Grimelius stain made the diagnosis of carcinoid and secretion of peptide hormones by any of these granulosa cell tumors less likely.

Nouwen et al., 1985 demonstrated immunohistochemically the presence of HPLAP in benign, borderline and malignant ovarian tumors including a well differentiated and a poorly differentiated granulosa cell "carcinoma" (sic). This discrepancy could be the result of difference in histological criteria used for diagnosis of granulosa cell tumors. In contrast to their results the 6 granulosa cell tumors in our series tested for HPLAP were negative.

None of the remaining tumor markers were positive in any of the granulosa cell tumors tested. Therefore serological estimation of these markers appears to be of no value in the follow-up

of these patients.

This study indicates the value of the use of antibodies directed against intermediate filament proteins and EMA to distinguish granulosa cell tumors from undifferentiated ovarian carcinomas. Therefore their use should complement the routinely used histological techniques in making such a distinction when necessary. This is worthwhile considering the much better prognosis of granulosa cell tumors.

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Addendum to Chapter 5

The immunohistochemical marker profile and Grimelius staining results of granulosa cell tumors and tumors to be considered in differential diagnosis.

Tumors	Ker.	Vim.	EMA	CEA	AFP	Grim.
Granulosa cell tumors (n=39)	-	+	-	-	-	-
Juvenile granulosa cell tumor (n=1)	-	+	-	-	-	-
Undifferentiated carcinoma (n=6)	+	-	++	+/-	-	-
Sertoli-Leydig cell tumor (n=2)	+*	+/-	-	-	+/-**	-
Carcinoid (n=2)	+	-	-	-	-	+
Small cell carcinoma (n=2)	-	-	-	-	-	-
Endometrioid stromal sarcoma (n=1)	-	+	-	-	-	-

Ker: keratin, Vim: vimentin, EMA: epithelial membrane antigen, CEA: carcinoembryonic antigen, Grim: Grimelius, AFP: alpha-fetoprotein. Tests were carried out on paraffin embedded tissue.
n = number of tumors examined.

* = denotes staining of tubular structures lined by epithelial-like cells representing Sertoli cell differentiation.

** see Chadha S, Honnebier WJ & Schaberg A. Raised serum alpha-fetoprotein in Sertoli-Leydig cell tumor (androblastoma) of ovary: Report of two cases. Int J Gynecol pathol 1987;6:82-88.

CHAPTER 6

IMMUNOHISTOCHEMICAL STUDY OF STEROIDS IN OVARIAN GRANULOSA CELL TUMORS: CORRELATION WITH ULTRASTRUCTURE AND CLINICAL MANIFESTATIONS

Introduction

Granulosa cell tumors (GCT) are the most common type of ovarian neoplasms associated with endocrine manifestations: i.e. approximately three quarters are accompanied by symptoms of hyperestrinism (Young & Scully, 1984; Fox, 1985). Rarely these tumors may be virilising (Norris & Taylor, 1969; Giuntoli et al., 1976; Wilansky, Scott & Lachange, 1976; Jarabak & Talerman, 1983). Women with a granulosa cell tumor also have a significantly higher risk of developing endometrial carcinoma which is attributed to hyperestrinism (Fox, Agrawal & Langley, 1975; Scully, 1977).

Localisation of steroid hormones in ovarian gonadal stromal tumors has been extensively studied immunohistochemically (Kurman et al. 1978; Kurman, Goebelsmann & Taylor, 1979; Kurman, Ganjei & Nadji, 1984). Their results showed that in granulosa theca tumors estradiol is localised mainly in granulosa cells suggesting that granulosa but not theca cells are primarily responsible for estrogen production in granulosa theca cell tumors.

Ultrastructural studies have also shown that in granulosa cell tumors only a small population of tumor cells possess large tubulovesicular mitochondria and a moderate to large amount of abundant smooth endoplasmic reticulum, organelles believed to be associated with steroid production (Gaffney et al., 1983).

The aim of this study was to assess the feasibility of immunohistochemical detection of steroids (estradiol, progesterone and testosterone) in paraffin embedded material. Therefore a number of these tumors were also studied ultrastructurally and the immunohistochemical results were correlated with the endometrial findings in these same patients.

Materials and Methods

Materials

From the files of the Dutch Ovarian Tumor Committee 17 granulosa theca cell tumors were retrieved. Fourteen cases originally diagnosed as granulosa cell tumors and referred from the regional hospitals to the Rotterdam Radiotherapy Institute were also included in this study. This series comprised granulosa cell tumors with and without clinical evidence of estrogen secretion. Clinical information and follow-up was available from all these cases. Formalin fixed and routinely paraffin embedded tissue blocks were available in all above cases. The final histological diagnosis was based on sections stained with hematoxylin azofloxin using standard criteria (Scully, 1979) and immunohistochemical reaction pattern with antibodies to intermediate filament proteins and epithelial membrane antigen (chapter 5).

Electron microscopy was performed on formalin fixed tissue material from 12 cases and frozen tissue from 2 cases.

In addition frozen specimens from two granulosa cell tumors were analysed by radioimmunoassay (RIA).

Methods

Antibodies: The mouse monoclonal antibodies and the rabbit antisera used in the present study are listed in Table I.

Table I Commercial source of antibodies used

Antibodies	Commercial source	Dilution
rabbit anti-estradiol	Ortho Diagnostics	kit
rabbit anti-testosterone	Ortho Diagnostics	kit
rabbit anti-progesterone	Diagnostic Product Corp.	several
mouse anti-progesterone	Unipath	several
rabbit anti-progesterone	Chemicon	several

To detect the binding of mouse monoclonal antibodies on tissue sections we applied the indirect conjugated method using a

rabbit anti-mouse antiserum conjugated to horse-radish peroxidase (RAM-PO) purchased from DAKO, Denmark. The binding of rabbit antibodies was detected by a horse-radish peroxidase conjugated swine anti-rabbit antiserum (SWAR-PO) purchased from DAKO, Denmark.

Immunohistochemistry

Paraffin sections of 5µ thickness were deparaffinized in xylene and absolute ethanol. The endogenous peroxidase activity was blocked by incubation in methanol containing 3% hydrogen peroxide for 30 minutes at room temperature. Before the application of primary rabbit antisera reduction of nonspecific background staining was achieved by incubation of the sections with 20% nonimmune swine serum (NSS) diluted in phosphate buffered saline (PBS) for 15 minutes. The sections were then overlaid with the appropriate dilutions of the primary antibodies for 1 hour at room temperature, rinsed for 10 minutes with PBS, incubated for another 30 minutes with the second (conjugated) antibody and rinsed again in PBS for 10 minutes. The RAM-PO was diluted 1:100 in PBS containing 5% nonimmune human serum (NHS) and 5% nonimmune rabbit serum (NRS). The SWAR-PO was diluted 1:100 in PBS containing 5% NHS and 5% NSS. Antibody localisation was visualised by incubation of sections with a TRIS-buffered saline solution (0.05 M, pH 7.4) containing 50 mg % 3,3 diamino-benzidine-tetrahydrochloride (Fluka, FRG) and 0.03% hydrogen peroxide. Slides were then washed with running tapwater, counter-stained with hematoxylin and mounted in Malinol.

Immunohistochemistry was also performed on snap frozen tissues from 3 GCT. Cryostat sections of 5µ thickness were air-dried thoroughly for at least 30 minutes at room temperature and fixed in acetone for 10 minutes. The remaining procedure was identical to that described for paraffin sections.

Positive controls for the detection of estradiol, progesterone and testosterone consisted of sections of ovaries containing follicles, corpora lutea and testis, respectively.

Electron microscopy

Fourteen GCT were examined by electron microscopy. Twelve tumors had prior fixation in 10% formaldehyde and from two cases frozen tumor tissue available was diced in 1 to 2 mm cubes and placed in 4% formaldehyde and 1% glutaraldehyde. All tumor tissue intended for electron microscopic examination was washed in phosphate buffer by Millonig and postfixed in 1% osmium tetraxide. Samples were dehydrated through graded alchohols and embedded in Epon.

Semithin sections (0.5 to 1 µm) were stained in toluidine blue and selected blocks were thin sectioned with diamond knives, mounted on 200 mesh grids and contrasted with uranyl acetate and lead citrate. Grids were examined in a Philips EM 201 electron microscope.

Determination of tissue steroid level

To evaluate the significance of the immunohistochemical reactions for steroid hormones, particularly estradiol, a few ovarian tumors (2 granulosa cell tumors, 1 thecoma and an ovary with hyperthecosis) were tested by means of a RIA before and after dehydration. These determinations were performed directly on snap frozen tissue as well as after thawing the same tissue in buffered formalin, dehydration in alchohol and storage in toluene for 24 hours. The toluene was then allowed to evaporate (room temperature). For RIA the tissues were cut into small pieces (1 mm3), homogenised in glass-glass potter-type homogenisers and finally sonicated. Acetone was added to precipitate proteins and the acetone water layer was dried until only water remained. The water layer was extracted with ether and finally ether was evaporated. To the residue 1 ml of estradiol-free human serum was added and the sample was tested in a RIA using kits commercially obtained from Diagnostics Product Corporation (DPC), Los Angeles, CA.

Results

Table II shows the clinical and pathological findings in 25 patients with GCT and 6 with a carcinoma.

Light microscopy

Twenty-five of the 31 neoplasms had the histological appearance characteristic of a granulosa cell tumor. Fifteen of these neoplasms were pure granulosa cell tumors with in ten a minor theca cell component. The granulosa cells had scanty cytoplasm and round or ovoid nuclei often with indented nuclear membrane suggestive of a "coffee-bean" appearance. In almost all tumor cells a small nucleolus was seen. A variety of histological patterns were present, the diffuse pattern being the most frequently observed. Call-Exner bodies were seen primarily in the microfollicular pattern as small spaces containing dense eosinophilic material or nuclear fragments. The remaining 6 neoplasms were undifferentiated carcinomas composed of solid areas lacking coffee-bean nuclei. These 6 neoplasms were vimentin negative, keratin and epithelial membrane antigen (EMA) positive and were therefore reclassified as undifferentiated carcinomas (chapter 5).

Steroid localization

Twenty-four of the 25 GCT tested for estradiol showed positive staining (Fig. 6.1) predominantly in the granulosa cells but also in theca cells. The 6 undifferentiated carcinomas were negative for estradiol. All the 25 tumors tested for progesterone were positive (Fig. 6.2). Sixteen of the 25 tested for testosterone were only moderately positive (Fig. 6.3) compared to estradiol and progesterone immunohistochemistry. The stromal component also showed staining by antisera directed against the 3 steroids though in most tumors this staining was less intense than that of the granulosa cells.

Table II

Clinical and Pathological findings in 31 patients with ovarian neoplasms primarily diagnosed as granulosa cell tumor

Pat. no.	Age (yrs)	Tumor diam.	LM IM	IM: Estradiol	IM: Progesterone	IM: Testosterone	IM: Endometrium	Pat. status
1	37	?	Mi	++	+	+		A 132 mo
2	47	7.5	Di	+	+	-		Lfu 32 mo
3	58	3.5	Mi	++	++	++	Ca.	Lfu 4 mo
4	58	?	Di	+	+	-	Hyper.	A 132 mo
5	50	20	Di	+	+	-		Lfu 14 mo
6	39	5	Ms	+	+	-		A 132 mo
7	53	12	Tr+Di	±	+	-	Prol.	A 132 mo
8	20	5	Ma	++	+	+		A 72 mo
9	51	10	Tr	+	+	-	No Prol.	A 60 mo
10	53	5.5	Tr+Di	+	+	-	Prol.	A 60 mo
11	75	4	Tr+Di	+	+	-		D 39 mo ht dis
12	32	6	Mi	++	+	-		A 29 mo
13	52	8	Di	+	+	+		A 228 mo
14	61	7	Di	+	+	+	Hyper.	A 48 mo
15	61	24	Di	+→++	+	-	Hyper.	A 32 mo
16	27	7.5	Mi	+→++	+	-		A 45 mo
17	66	8	Di	+	+	++	Hyper.	A 135 mo
18	46	12	Tr+Di	+	+	+	No prol.	A 72 mo
19	37	16	Tr+Di	±	+	++		A 144 mo
20	68	?	Di	+	+	+		D 12 mo rec
21	58	10	In+Tr +Mi	+	+	+	Hyper.	A 85 mo
22	62	10	Tr+Di	+	+	++	Hyper.	D 39 mo rec
23	51	9	Mi	+	+	+	Hyper.	D 46 mo lung ca

Pat. no.	Age (yrs)	Tumor diam.	LM IM	IM: Estradiol	IM: Progesterone	IM: Testosterone	IM: Endometrium	Pat. status
24	54	22	Tr	-	+	-		D 98 mo rec
25	56	?	Di	+	+	+	Hyper.	A 60 mo
26	41	14.5	Ca	-	+	+		D 50 mo ca
27	41	?	Ca	-	+	+		D 10 mo ca
28	69	?	Ca	-	+	+		D 14 mo ca
29	73	?	Ca	-	+	-		D 8 mo ca
30	74	?	Ca	-	-	-		D 27 mo ca
31	53	?	Ca	-	+	-		D 39 mo ca

LM = light microscopy; IM = immunohistochemistry; + → ++ denotes intensity of positive reaction; Mi = micro-follicular; Di = diffuse; Tr = trabecular; Ma = macrofollicular; In = insular; Ms = moire silk; Ca = undifferentiated carcinoma; A = alive; D = dead; Lfu = lost to follow-up; mo = months; hyper = hyperplasia; Prol. = proliferation; rec = recurrence; ht dis = heart disease.

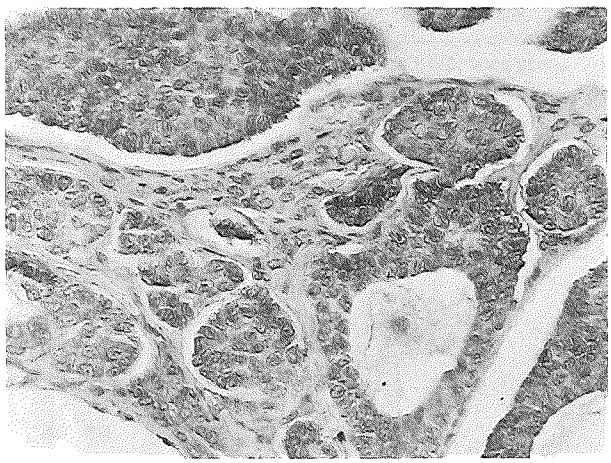

Fig.6-1
Granulosa cell tumor - immunoperoxidase - estradiol showing diffuse positivity of granulosa cells but also theca cells with high background staining (x380)

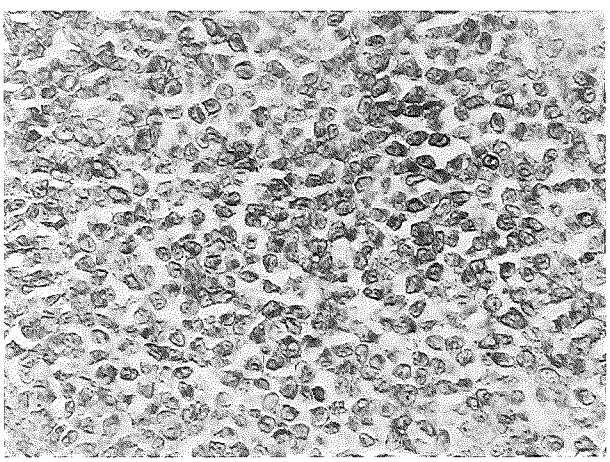

Fig.6-2
Granulosa cell tumor - immunoperoxidase - progesterone showing diffuse positivity of granulosa cells. (x380)

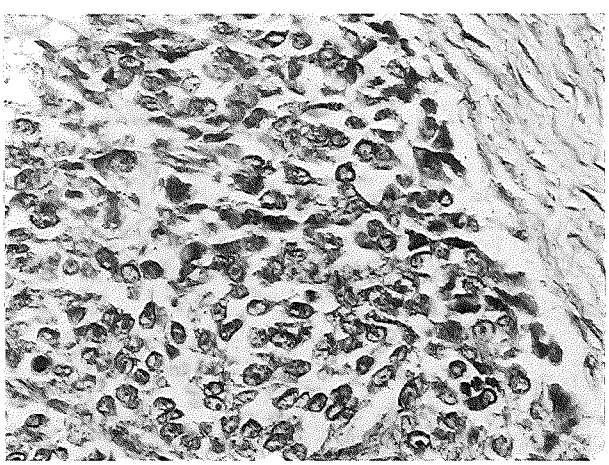

Fig.6-3
Granulosa cell tumor - immunoperoxidase testosterone showing positivity of granulosa cells but also stroma cells. (x380)

Effect of tissue dehydration in tissue processing on estradiol content in ovarian tumors

Determination of estradiol was performed by RIA on ovarian tissues (see Methods). The results of these experiments are shown in Table III. The results indicate that in all cases the dehydration process leads to a drastic reduction in estradiol content below the detection level of RIA.

Table III Effect of tissue dehydration on estradiol content.

	Estradiol content (pmol/gram)	
	not dehydrated	dehydrated
Hyperthecosis	120	< 0.35
Thecoma	0.37	< 0.23
Granulosa cell tumor I	3.3	< 0.04
Granulosa cell tumor II	1.8	< 0.02

Associated endometrial changes

In 13 cases endometrium was available for examination. In 8 patients hyperplasia of endometrium was present and in 1 an adenocarcinoma was detected. In 2 cases the endometrium was in the proliferative phase. Table IV shows correlation of endometrial findings with steroid localisation.

Table IV Correlation of immunohistochemical results and endometrium findings.

Endometrium findings	Estradiol	Testosterone	Progesterone
Endometrial hyperplasia	8/8	6/8	8/8
Endometrial adenocarcinoma	1/1	1/1	1/1
No Hyperplasia	3/4	4/4	4/4

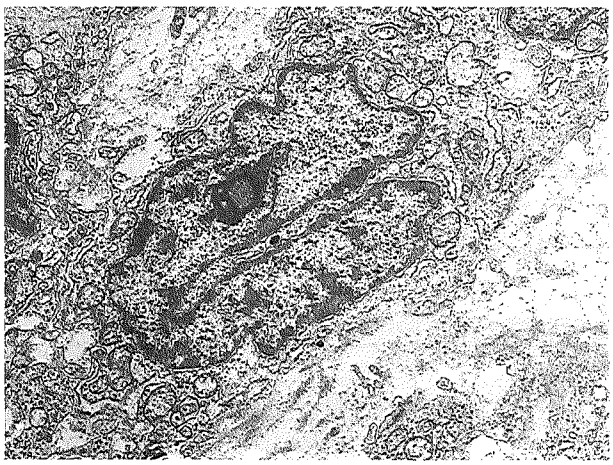

Fig.6-4
Granulosa cell tumor. Nucleus showing typical single deep indentation. x4000

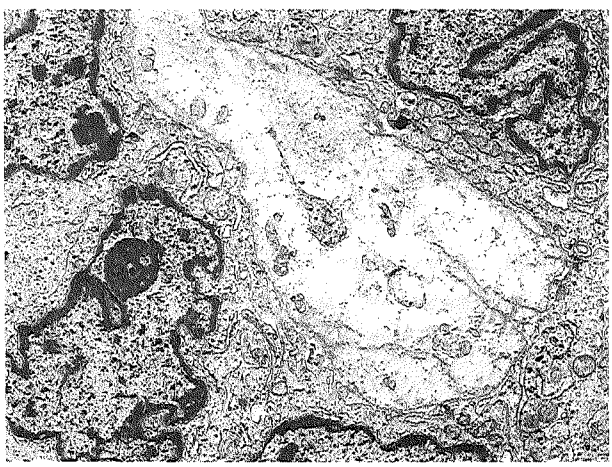

Fig.6-5
Granulosa cell tumor. Typical Call-Exner body surrounded by granulosa cells with indented nuclei. Call-Exner body is defined by a basal lamina and contains basal lamina like material. x4000

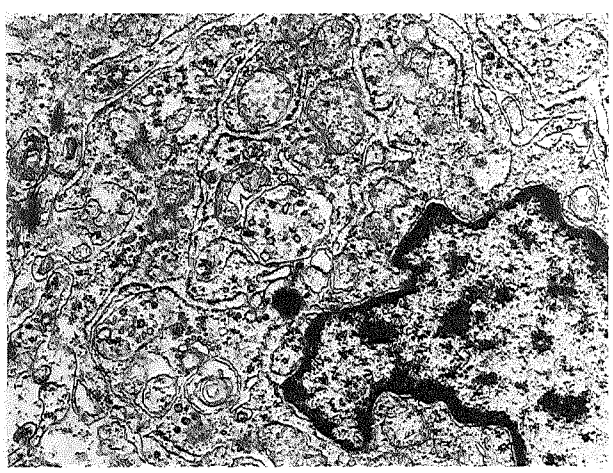

Fig.6-6
Granulosa cell tumor. Part of cytoplasm of a tumor cell containing abundant smooth endoplasmic reticulum, mitochondria with tubular cirstae. x8000

Electron Microscopy

Fourteen granulosa cell tumors were examined by electron microscopy. The quality of the electron micrographs from the tumors varied considerably depending on the duration of preservation in formalin. Electron micrographs of frozen tumors were considerably better. In general the tumor cells were polygonal with ovoid nuclei showing a typical single longitudinal indentation corresponding to the coffee-bean appearance (Fig. 6.4). The nuclei showed marginal condensation of chromatin with one or more nucleoli. The tumor cells had a scanty cytoplasm, wide intercellular spaces and tight junctions were present. The cytoplasm in most cases contained only few organelles. Call-Exner bodies were identified focally as hollow intercellular spaces surrounded by neoplastic cells (Fig. 6.5). These intercellular spaces were limited by a peripheral basal lamina and contained basal lamina like material, cellular fragments and amorphous debris. In 2 cases 1-15% of cells were characterised by moderate to abundant smooth endoplasmic reticulum and larger mitochondria with tubular cristae considered to be characteristic of steroid production (Fig. 6.6).

Discussion

We have studied 31 ovarian neoplasms primarily diagnosed as granulosa cell tumors for the presence of estradiol, progesterone and testosterone. Six of these 31 tumors were later reclassified as undifferenatiated carcinomas on the basis of morphology and immunohistochemical reaction pattern with antibodies to intermediate filament proteins and epithelial membrane antigen (chapter 5).

Twenty-four of the 25 granulosa cell tumors stained for estradiol predominantly in granulosa cells but also in theca cells. In a number of papers Kurman and his colleagues (1978, 1979, 1984) reported the immunohistochemical localisation of steroids in gonadal stromal tumors. These authors came to the conclusion that judging from the relative intensity of staining

reaction granulosa cells and not the theca cells are primarily responsible for estrogen production. They stated, however, that a positive reaction could either be based on steroid synthesis, intracellular storage or binding to specific hormone receptors. The results of above authors as well as our results can therefore be considered as an argument in favour of the feasibility of this technique.

We also noticed a high background staining similar to that reported by the above authors which could be attributed to the solubility of steroid hormones leading to ready diffusion throughout the tissue. Also cross reactions between antisera against estradiol and testosterone due to closeness of their chemical structure may exist although these authors considered such a reaction unlikely and did not indicate the exact figures for these cross reactions.

A severe drawback of the commercially obtained antisera is that multiple batches of antisera tested showed highly variable results varying from strongly positive to negative. Concerning the specificity of antisera directed against progesterone and testosterone it was striking that 5, respectively 3 undifferentiated carcinomas showed a positive staining reaction. In addition the antisera directed against testosterone and progesterone also showed positive staining with other tissues e.g. intestinal epithelium (data not shown). Unfortunately the antisera used by Kurman et al. in their study are no longer available (personal communication).

The data derived from the biochemical estimation of tissue steroids show that organic solvents lead to a drastic reduction of estradiol content. The very low level of steroids even below detection level of RIA (Table III) remaining in the tissues after extraction by organic solvents is less probable to be detected by immunohistochemical technique. The diffuse staining pattern makes it even more unlikely that these minute amounts of steroids are detectable by immunohistochemical techniques. One possible explanation of positive immunohistochemical results could be the presence of a protein bound fraction of steroids which cannot be extracted from the tumor tissue by the method employed for RIA.

This possibility is unlikely in view of the results of De Jong, Hey & Van der Molen (1974) who showed that after the extraction procedure as employed in this study only 7% of estradiol remained in testicular tissue containing estradiol receptors. Another possibility is that the antiestradiol antiserum used is directed against antigenic determinants shared by the estradiol-protein complex, used as immunogen and another (non-related) tissue protein.

The above data obtained from RIA on dehydrated tissues contradict the opinion of Kurman and his colleagues who stated that sufficient steroids remain to permit detection by immunohistochemical techniques. To our knowledge none of the previous authors have performed biochemical assays for tissue steroid level to confirm their immunohistochemical observations.

In our series endometrium findings were available in 13 of the 25 cases with GCT. Eight had endometrial hyperplasia, 1 an endometrial carcinoma and 2 cases had a proliferative endometrium. Kurman, Goebelsmann & Taylor (1979) studied 11 cases of granulosa theca cell tumors, 9 out of these had endometrial hyperplasia, in 1 an endometrium polyp was present and in 1 case no endometrium sample was available. In all 11 tumors these latter authors found steroid localisation in granulosa cells. While Gaffney et al. (1983) analysed 11 granulosa cell tumors, 6 of their cases had hyperplasia of endometrium, 2 cases showed a proliferative endometrium, and 1 case an endometrium polyp. From the remaining 2 cases no endometrium sample was available. These latter authors demonstrated estradiol localisation in 1 to 15% of tumor cells in 10 cases and in the remaining 1 case the estradiol staining was equivocal. In our series a positive staining reaction for estradiol did not correlate well with clinical evidence of hyperestrinism (Table IV). This apparent lack of biological significance of immunohistochemical staining reactions for estradiol may be explained in three ways:

1. In reality what we detect is some cross reacting compound which differs from estradiol.
2. The endometrium is hormone refractive in a proportion of the patients.
3. Estradiol present in these GCT is not secreted in sufficient amounts in order to lead to the clinical manifestation. The latter explanation seems to be highly unlikely as steroids can easily diffuse through cellular membranes.

In accordance with previous ultrastructural studies (McCaulay, Weliky & Schulz, 1967; Gondos, 1969; Toker, 1968; Pedersen & Larsen, 1970; Klemi & Gronroos, 1979; Gaffney et al., 1983; Salazar & Gonzalez-Angulo, 1984), we also observed that the GCT were predominantly composed of poorly differentiated cells. Only in 2 tumors a few cells (1-15%) were detected containing a moderate to abundant smooth endoplasmic reticulum and mitochondria with tubular cristae. These latter features were considered to be characteristic for steroid synthesis. One of both patients showed endometrial hyperplasia and the other an actively proliferating endometrium after menopause.

In conclusion the data of this study cast serious doubt on the feasibility of the immunohistochemical technique for detection of steroids on paraffin-embedded tissues. This is strengthened by the observation that no clear-cut relationship exists between immunoreactivity of a granulosa cell tumor for estradiol and the presence of clinical signs of hyperestrinism.

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SUMMARY

Granulosa cell tumors (GCT) of the ovary account for 2% of all ovarian tumors. As the name indicates, they are composed of granulosa cells but may also contain an admixture of theca cells. They are potentially malignant but, except for extraovarian spread, which is generally agreed to indicate poor prognosis other criteria to judge the prognosis remain in dispute.

The aim of this study was to examine a series of these tumors histopathologically, immunohistochemically and by flow cytometry in order to achieve a higher diagnostic accuracy and to ascertain which criteria are of prognostic value.

Sixty-one cases were studied. Fifty-one of these patients had been referred to the Netherlands Committee for Ovarian Tumors in the period 1966 to 1985 while 10 cases originally diagnosed as granulosa cell tumor and referred to the Rotterdam Radiotherapy Institute for treatment were retrieved. Follow-up of these patients to a maximum period of 19 years was available.

Even though there has been some measure of agreement relating to a few pathological features of GCT e.g. tumor diameter and rupture, attempts to correlate the histological pattern, degree of nuclear atypia and the mitotic activity have not been uniformly successful. Ten separate criteria were evaluated which from our own experience and review of the literature could be considered to be of prognostic value. These criteria are FIGO (International Federation of Gynecology and Obstetrics) stage at the time of operation, age at diagnosis, size of tumor, rupture of tumor, histological pattern, nuclear atypia as evaluated by degree of nuclear pleomorphism and hyperchromasia, mitotic activity, presence of lymphatic invasion and necrosis. The prognostic value of these factors was evaluated by analysing the effect of all these variables on the entire group of patients and on those with stage I disease. Survival curves were plotted according to Kaplan-Meier method. The features indicative of poor prognosis are: age above 40, FIGO stage at the time of diagnosis, rupture of tumor in stage I disease, tumor diameter above 5 cm, and degree of nuclear pleomorphism and hyperchromasia. The factors

which did not correlate with prognosis were mitotic activity, diffuse histological pattern, lymphatic invasion and mode of surgical treatment (chapter 3).

In addition, since a relationship has been shown between DNA ploidy and prognosis in malignant epithelial ovarian tumors and malignant tumors of other organs, we studied if this parameter may be of importance in GCT. Eighty-eight percent (38) of the tumors originally diagnosed as GCT were DNA diploid or near-diploid while 12% (5) of the tumors were DNA aneuploid in sharp contrast to the high aneuploidy reported in malignant epithelial ovarian tumors. Moreover the 5 DNA aneuploid tumors in our series included 3 carcinomas (on the basis of immunohistochemical staining reactions with antibodies to intermediate filament proteins) which had been primarily incorrectly diagnosed as GCT. Therefore an undifferentiated carcinoma should be considered in the differential diagnosis of granulosa cell tumors with high DNA aneuploidy. The results also suggest that the presence of an aneuploid stemline is associated with a poor prognosis. (chapter 4).

Though most GCT with the typical morphological features e.g. coffee-bean nuclei and Call-Exner bodies may be diagnosed with ease, in some cases (as noted above) a correct diagnosis may be difficult mainly due to the various histological patterns and combinations thereof. The differential diagnosis of GCT includes undifferentiated carcinoma, small cell carcinoma with hypercalcemia, Sertoli-Leydig cell tumor, carcinoid and endometrioid stromal sarcoma.

It appears from this study that an undifferentiated carcinoma can in particular be difficult to distinguish from a histologically diffuse type of GCT. Specially useful in making this distinction is the demonstration of antibodies directed against intermediate filament proteins, vimentin and cytokeratin as well as epithelial membrane antigen. Therefore, their use should complement the routinely used histological techniques in making such a distinction, a worthwhile proposition considering the much better prognosis of GCT. In our experience epithelial membrane antigen appears to be a better marker for ovarian epithelial

tumors than cytokeratin. No other tumor marker was identified in GCT (chapter 5).

More than two thirds of GCT are functionally active and along with theca cell tumors they comprise the group most frequently associated clinically with endocrine manifestations. We examined 25 GCT and 6 undifferentiated carcinomas originally diagnosed as GCT immunohistochemically for the presence of estradiol, progesterone and testosterone in paraffin embedded tumor tissue.

Twenty-four of 25 GCT stained for estradiol predominantly in granulosa cells but also in theca cells while none of the carcinomas were positive. All 25 GCT and 5 out of 6 carcinomas were positive for progesterone while 13 GCT and 3 carcinomas stained moderately for testosterone.

The positive staining for estradiol in GCT in our series did not correlate well with clinical signs of hyperestrinism.

Similarly, no correlation was seen between the latter and ultrastructure: i.e. only 1-15% of cells had smooth endoplasmic reticulum, larger mitochondria with tubular cristae, features generally associated with steroid synthesis.

To evaluate the significance of the immunohistochemical reaction for estradiol a radioimmuno-assay (RIA) was performed for estradiol on a few estradiol containing tumors before and after dehydration. Since the organic solvents led to a drastic reduction of estradiol content below the detection level of RIA our results indicate that immunohistochemical staining for steroids of paraffin embedded GCT may be of limited value (chapter 6).

SAMENVATTING

Granulosa cel tumoren (GCT) van het ovarium vormen 2% van alle ovariumtumoren. Zoals de naam al zegt, zijn deze tumoren opgebouwd uit cellen met het karakter van granulosa cellen; ze kunnen daarbij ook theca cellen bevatten. GCT zijn potentieel maligne. Behalve uitbreiding buiten het ovarium, waarvan algemeen wordt aangenomen dat het een slechte prognose heeft, zijn andere criteria om de prognose te beoordelen een punt van discussie.

Het doel van deze studie was om een serie GCT histopathologisch, immuunhistochemisch en met behulp van flow cytometrie te onderzoeken, om zodoende tot een betere diagnostiek te komen en om vast te stellen welke criteria van prognostisch belang zijn.

Een en zestig gevallen werden onderzocht. Een en vijftig van deze patienten waren verwezen naar de Nederlandse Ovarium Tumoren Commissie in de periode tussen 1966 en 1985, terwijl 10 gevallen, oorspronkelijk gediagnostiseerd als GCT, doorgestuurd waren naar het Rotterdams Radiotherapeutisch Instituut. De follow-up van de hele groep patienten bedroeg maximaal 19 jaar.

Hoewel er in de literatuur een zekere mate van overeenstemming bestaat betreffende enkele prognostiche kenmerken van GCT, zoals tumorgrootte en tumorruptuur, zijn pogingen om het histologisch patroon, de mate van kernatypie en de mitotische activiteit te correleren met de prognose niet even succesvol geweest. Tien verschillende criteria, die uit eigen ervaring en uit een overzicht van de literatuur, van prognostisch belang leken te zijn, werden geëvalueerd. Deze criteria zijn: FIGO (Internationale Federatie van Gynaecologie en Obstetrie) stadium ten tijde van operatie, leeftijd op het moment van diagnose, afmetingen van de tumor, ruptuur van de tumor, histologisch patroon, kernatypie beoordeeld aan de hand van de mate van kernanisomorfie en hyperchromasie, mitotische activiteit, aanwezigheid van invasieve groei in lymfbanen en necrose. De prognostische betekenis van deze factoren werd geëvalueerd, door de relatie van al deze variabelen tot de prognose binnen de hele groep patienten en bij de patienten met tumoren in stadium I, te analyseren. Overlevingscurven werden uitgezet volgens de Kaplan-

Meier methode. Factoren welke een slechte prognose aangeven zijn: leeftijd boven de 40 jaar, FIGO stadium ten tijde van de diagnose, ruptuur van de tumor in stadium I, tumordiameter groter dan 5 cm en mate van kernanisomorfie en hyperchromasie. Factoren die niet bleken te correleren met de prognose zijn: mitotische activiteit, diffuus histologisch patroon, lymfbaan invasie, en wijze van chirurgische behandeling (hoofdstuk 3).

Gezien de relatie tussen ploidie en prognose bij maligne epitheliale ovarium tumoren en bij maligne tumoren van andere organen, is gekeken of deze parameter ook van betekenis is bij GCT. Achtentachtig procent (38) van de tumoren oorspronkelijk gediagnoseerd als GCT waren DNA diploid of bijna-diploid, terwijl 12% (5) van de tumoren DNA aneuploid waren, dit in tegenstelling tot de hoge frequentie van aneuploidie beschreven bij maligne epitheliale ovarium tumoren. Evenwel in onze serie waren er onder de 5 aneuploide tumoren 3 carcinomen (op basis van immunohistochemische kleuringen met antistoffen tegen intermediaire eiwitten) die aanvankelijk verkeerd waren gediagnostiseerd als GCT. Bij de differentiële diagnose van GCT met hoge DNA aneuploidie, dient dan ook het ongedifferentieerde carcinoom te worden opgenomen. De resultaten van het onderzoek suggereren bovendien dat de aanwezigheid van een aneuploide stamlijn samen gaat met een slechte prognose. (hoofdstuk 4).

Hoewel de meeste GCT op grond van typische histologische kenmerken zoals koffieboon-kernen en Call-Exner lichaampjes, gemakkelijk kunnen worden gediagnostiseerd, kan in sommige gevallen (zoals hierboven vermeld) het stellen van de juiste diagnose problemen opleveren. De differentiële diagnose van GCT omvat ongedifferentieerd carcinoom, kleincellig carcinoom met hypercalcaemie, Sertoli-Leydig cel tumor, carcinoid en endometrioid stroma sarcoom. Uit deze studie blijkt dat vooral het ongedifferentieerde carcinoom moeilijk te onderscheiden kan zijn van het diffuus type GCT. Zeer bruikbaar om deze twee tumortypen te onderscheiden is het aantonen van antilichamen gericht tegen intermediair-filament eiwitten (vimentine en cytokeratine), en tegen epitheliaal membraan antigeen (EMA). Tegen deze achtergrond wordt het dan ook raadzaam geacht om deze

laatstgenoemde technieken bij het routine histologische onderzoek uit te voeren, vooral gezien de betere prognose van GCT. Onze ervaring is dat het epitheliaal membraan antigeen een betere marker voor epitheliale tumoren van het ovarium is dan cytokeratine. Voor de diagnose van GCT kon geen andere marker aangewezen worden (hoofdstuk 5).

Meer dan twee-derde van de GCT zijn functioneel actief en tesamen met de theca-cel tumoren vormen ze de groep die klinisch het meest frequent gepaard gaat met endocriene uitingingen. In paraffine ingebed weefsel van 25 GCT en van 6 ongedifferentieerde carcinomen, aanvankelijk gediagnostiseerd als GCT, is immuunhistochemisch onderzocht op aanwezigheid van oestradiol, progesteron en testosteron. Bij 24 van de 25 GCT bleken voornamelijk de granulosa cellen, maar ook de theca-cellen, positief met anti-oestradiol, terwijl geen van de carcinomen positief waren. Alle 25 GCT en 5 van de 6 carcinomen waren positief met anti-progesteron terwijl 13 GCT en 3 carcinomen matig aankleurden met anti-testosteron.

De positieve reactie met anti-oestradiol bij GCT in onze serie, correleerde niet goed met de klinische symptomen van een verhoogde oestrogeen spiegel. Eveneens werd geen correlatie tussen een positieve reactie met anti-oestradiol en de ultrastructuur van de tumor cellen gezien: dat wil zeggen slechts 1-15% van de cellen had glad endoplasmatisch reticulum en grote mitochondrieën met tubulaire cristae, kenmerken welke in het algemeen worden beschouwd te wijzen op steroid hormoon synthese.

Om de betekenis van de immuunhistochemische reactie voor oestradiol te evalueren werd een radioimmuno-assay (RAI) voor oestradiol verricht op enkele oestradiol bevattende tumoren vóór en na dehydratie. Aangezien het oplossen van organische stoffen bij dehydratie tot een drastische verlaging van oestradiol, tot onder het detectie niveau van RIA leidde, lijkt de immuunhistochemische reactie op steroiden bij in paraffine ingebedde GCT van beperkte waarde (hoofdstuk 6).

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