Head and Neck Paragangliomas characteristics of tumour biology

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Chapter 1

Introduction and outline of the thesis

1. The paraganglion system

Anatomy and topography

The paraganglia are anatomically widely dispersed cell clusters of neuroectodermal origin that are associated with the autonomous nervous system and are capable of synthesising catecholamines.

The largest paraganglion is the adrenal medulla, an important neuroendocrine organ that is primarily involved in orthosympathetic regulation. Besides this adrenal station there are numerous extra-adrenal paraganglia that are dispersed along the body axis and located in the proximity of ganglia of the sympathetic chain (hence termed *para*ganglia) or in association with extensions of cranial nerves and blood vessels (in the head and neck-region and mediastinum).[1,2]

Based on histological similarities between different paraganglia, Kohn introduced the concept of a unitary system linking the adrenal medulla with the extra-adrenal paraganglia in 1903.[3] In Kohn's concept the adrenal medulla was regarded as a separate entity whereas the extra-adrenal system was divided in two major components; A) one associated with the orthosympathetic nervous system, including para-aortic, thoracic and abdominal paraganglia and B) the other component, that is associated with the parasympathetic nervous system and includes head and neck paraganglia and mediastinal locations.

The subsequent recognition of distinct differences in anatomic distribution, physiological function, innervation and microscopic anatomy resulted in further refinement of the original concept by Glenner and Grimley with the introduction of four interrelated families of extra-adrenal paraganglia.[4] These families include the *1)* branchiomeric paraganglia, which are situated in the head and neck and mediastinum, *2)* the intravagal paraganglion, *3)* aorticosympathetic paraganglia, extending axially and segmentally along the aorta including the organ of Zuckerkandl, and *4)* viscero-autonomic-paraganglia. (Figure 1.1)

Head and Neck Paraganglia
As mentioned previously, the paraganglia in the head and neck are primarily classified as branchiomeric paraganglia and are associated with the parasympathetic nervous system.

The most consistent paraganglion in the head and neck is the carotid body that is located at the carotid bifurcation. The carotid body serves as an afferent peripheral

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PARAGANGLION SYSTEM

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                    PARAGANGLION SYSTEM
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        ┌───────────────────────────────────────┐
EXTRA ADRENAL PARAGANGLIA              ADRENAL MEDULLA
        │
 ┌──────────────────┐
Parasympathetic          Orthosympathetic
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A. branchiomeric paraganglia	C. para-aortic paraganglia
• tympanic	• *Zuckerkandl* organ
• jugular	• para-aortic bodies
• carotid*	
• laryngeal	D. viscero autonomic paraganglia
• coronary	• retroperitoneal
• aortico pulmonary*	• bladder
B. intravagal paraganglia	
• vagal*	

Figure 1.1: topography and classification of the paraganglion system according to Kohn[3] and Glenner & Grimley.[4] *) = chemoreceptor-function.

chemoreceptor, that predominantly registers arterial oxygen concentration and transmits sensory signals through a branch of the glossopharyngeal nerve (Herring's nerve) towards the central nervous system.[2]

Other head and neck paraganglia include: *a)* the jugulotympanic paraganglion that is situated at the jugular bulb and tympanic plexus of Jacobson's nerve in the floor of the middle ear, *b)* the intravagal paraganglion that is situated in the perineurium of the vagal nerve in proximity of the ganglion nodosum, and *c)* secondary locations in the larynx, trachea and the nasal cavity. (Figure 1.2)[2,5,6]

The aorticosympathetic and viscero-autonomic paraganglia as well as the adrenal medulla are associated with the orthosympathetic nervous system. (Figure 1.3)

Embryology and Development

The parenchymal cells of paraganglia arise from the neuroectodermal tissue of the neural crest and are thought to migrate along innervating nerves or vasculature

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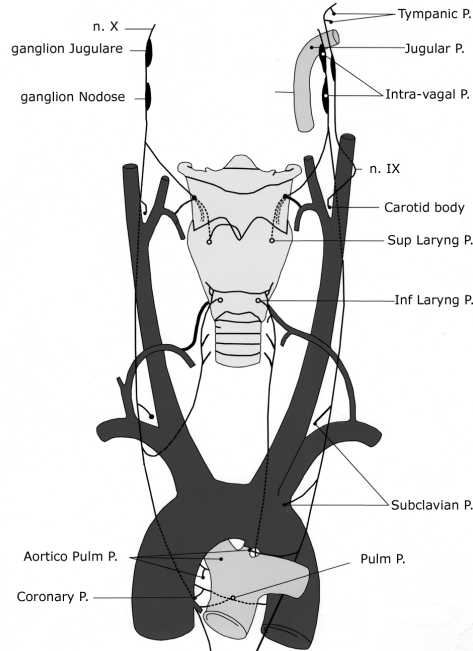
Figure 1.2: Distribution of the branchiomeric and intravagal paraganglia. (Courtesy of AFIP, Washington, 1974)[4]

towards their primordial location.[7] In case of the branchiomeric paraganglia, it is believed that pre-existing mesenchymal cells give rise to the fibrous stromal components.

Generally, the distribution of orthosympathetic paraganglionic tissues is quite extensive shortly after birth but regresses during early childhood coinciding with the maturation of the adrenal medulla. The branchiomeric and intravagal paraganglia are believed to decrease in some locations and to increase in others. The common head and neck paraganglia of the tympanic plexus and jugular bulb increase in numbers whereas the combined weight of both carotid bodies is associated with increasing body weight and age.[1,8]

The involutionary processes may explain the occasional development of ectopic paragangliomas in areas where no consistent paraganglia have been described. For instance, paraganglioma have been reported in the orbit, gallbladder, spermatic cord, vulva, ovaries and recently in the lungs.[9-12]

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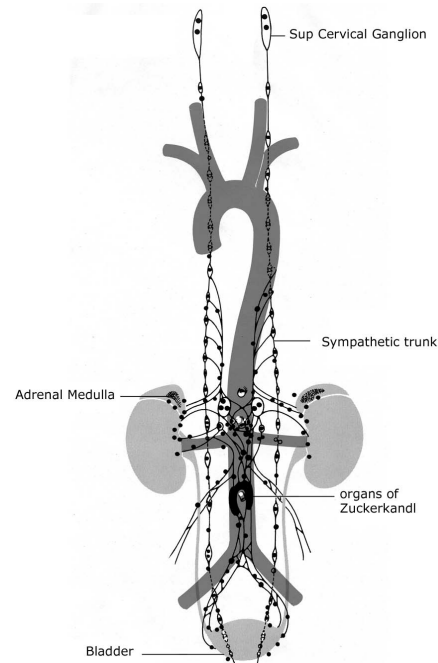
Figure 1.3: Distribution of aortico-sympathetic and viscero-autonomic paraganglia in a newborn child. (Courtesy of AFIP, Washington 1974)[4]

General Morphology and Histology

The branchiomeric head and neck paraganglia all have a similar morphology and histology. The best-studied head and neck paraganglion is the carotid body, which is grossly visible macroscopically as a flattened rice grain-shaped organ (5x5x2.5mm). It is situated medially in the adventitial plane of the carotid bifurcation and a fibrovascular pedicle (Mayer's ligament) may be seen carrying the small glomic arteries and myelinated nerve bundles. The majority of other head and neck paraganglia however, are microscopic structures that are composed of small aggregates of paraganglionic cells.

Microscopically, the carotid body is composed of multiple ovoid lobules separated by fibrous septa that contain abundant myelinated nerve fibres and small arteries that supply the individual lobules. Each lobule is organised in several nests of parenchymal chief cells (type I cells) and interspersing stroma that contains nerve endings, small arterioles and venules. At the periphery of the cell nests a second cell type, the sustentacular cell (type II cells), is present that is believed to have a supportive function. The typical nested architecture of chief cells and sustentacular

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cells is a prominent feature of branchiomeric paraganglia and termed *Zellballen*. (Figure 1.4)

Chief cells are round to oval, have a central nucleus with a granular eosinophillic cytoplasm. Three different forms of chief cells have been described in the carotid body: "light" cells, "dark" cells and pyknotic cells (also described as progenitor cells). The functional significance of these subtypes of chief cells remains unclear although changes in distribution of these subtypes may be associated with ageing and hyperplasia.[8,13]
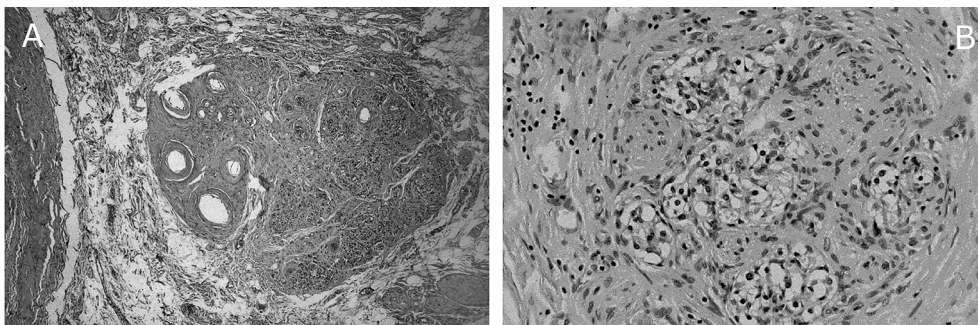
Figure 1.4: A) Section of a carotid body paraganglion situated in the adventitial plane of the carotid bifurcation (HE, 20x), **B)** Section of a carotid body paraganglion demonstrating the '*Zellballen*' architecture with clusters of chief cells surrounded by sustentacular cells at the periphery, situated in a highly vascularised stroma with unmyelinated nerve endings (HE 100x; colour image on page 139).

The sustentacular cells have a crescent nucleus and an elongated cytoplasm with slender extensions that envelope the chief cells and nerve axons, thus insulating small groups of chief cells from surrounding interstitial tissue and capillaries.

Chief cells are capable of synthesising and storage of catecholamines (norepinephrine, epinephrine and dopamine) and a number of neuropeptides and enzymes have been localized with various histochemical techniques.[1,14-16] Based on the reaction of catecholamines with chromates, paraganglia were traditionally divided in *chromaffin* and *non-chromaffin* under the light microscope, although these reactions are not always reliable and do not correspond with functional activity.[14,15] The branchiomeric and intravagal paraganglia are generally considered chromaffin negative whereas aorticosympathetic and viscero-autonomic paraganglia are generally chromaffin positive.

Sustentacular cells can be identified by immunohistochemical staining for the neurotrophic S-100 protein although in the normal paraganglion Schwann cells may display similar immunoreactivity.[17]

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Ultrastructure and chemogenic model

Ultrastructurally, light and dark chief cells have been identified, both containing abundant dense core neurosecretory granules indicative of their neuroendocrine nature.[18,19] The chief cells in the carotid body were found to have elongated extensions that abut and interdigitate in a complex fashion with surrounding chief cells as well as with cytoplasmatic processes of sustentacular cells confining them individually or in clusters, possibly representing functional units. As a result of the envelopment, the bodies of the sustentacular cells interpose between the chief cells and vessel walls, thus preventing direct contact of chief cells with the vascular channels. Besides enveloping the chief cells the sustentacular cells also unsheathe unmyelinated nerve fibres, conveying them from the periphery of the cell nests into direct contact with the chief cells. Based on synaptic morphology it is believed that the majority of nerve endings are afferent sensory fibres. Additionally some efferent fibres, presumably of sympathetic origin are also present within a chief cell unit.[20] (Figure 1.5)

Although the precise cellular mode of oxygen sensing is still not fully elucidated, it is generally believed that chief cells are the prime source of chemosensory activity in the carotid body.[21,22] Based on this assumption and ultrastructural observations, Grimley and Glenner conceived a model of chief cell units in the carotid body from
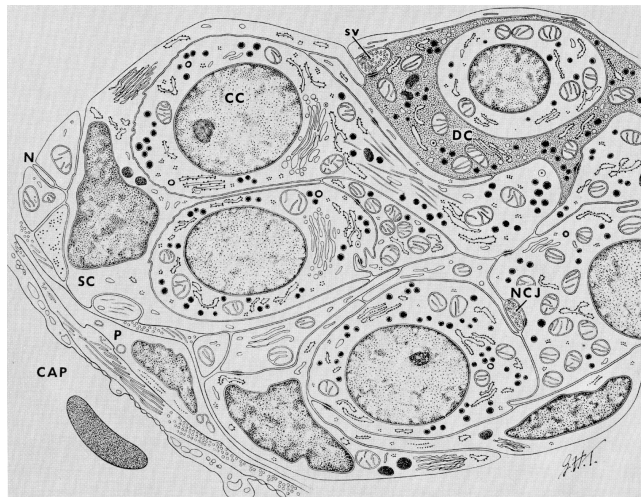

Figure 1.5: Schematic diagram displaying ultrastructure characteristics of a human carotid body. Chief cells (CC) containing dense neurosecretory granules are enveloped by sustentacular cells (SC). Unmyelinated nerve endings (NCJ) contact chief cells at special junctions. DC: dark variant chief cell, P: pericyte, CAP: capillary. (According to Grimley & Glenner).[18]

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which the chemogenic impulse is generated.[19] The units consist of chief cells that are circumscribed by the processes of sustentacular cells and capillary pericytes, thus being partially isolated from the interstitial stroma. According to this model, chief cells are sensitised resulting in local accumulation of neurotransmitters (presumably catecholamines) around afferent sensory fibres that subsequently results in depolarisation and convergence of action potentials in Herrings nerve. Organisation of chief cells would favour effective recruitment and accumulation of neurotransmitters in the vicinity of sensory nerve endings and facilitate interaction of chief cells in conjunction with signals from efferent sympathetic stimuli. Accordingly, the chief cells should be regarded as couples or transducers interposed between interstitium and nerve endings. (Figure 1.6)
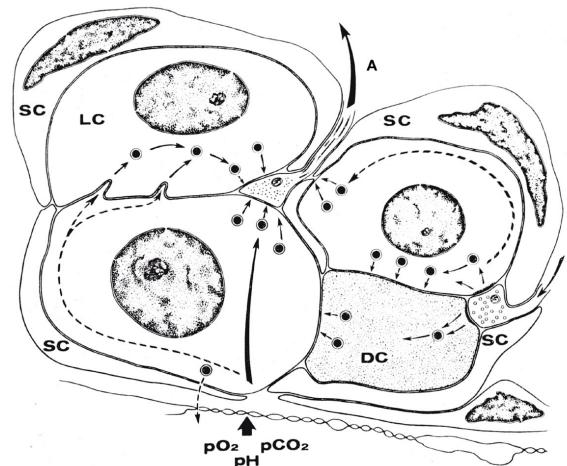
Figure 1.6: Schematic representation of the chemogenic unit in the carotid body according to Grimley & Glenner.[19] Arrows depict possible routes of chemogenic impulse transmission. Chief cells interact through direct depolarisation of their membranes (dotted arrows) or through release of catecholamines(solid arrows). Excitation of an afferent nerve fibre (A) presumably results from local release of bioamines at synaptic clefts. Efferent stimulation of chief cells may be established through synaptic release of bioamines from orthosympathetic nerve endings (E). *LC* & *DC*: light and dark-chief cell variants respectively, *SC*: sustentacular cell.

Hyperplasia of carotid body paraganglia

Several studies have reported the occurrence of hyperplasia of the carotid body under chronic hypoxemic or hypoxic conditions.[7,23] Hyperplasia in carotid bodies is characterised by an increase in number of parenchymal cells, often with a proliferation of other cell types as well.[17] Frequently, hyperplasia is accompanied with carotid body hypertrophy that usually occurs bilaterally and symmetrically.

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Criteria that define hyperplasia include increase in weight, size and increment in the percentage or differential count of elongated cells and chief cells and should be correlated with age related changes of carotid body morphology.[24,25] Microscopically, an increase in the number of lobules was observed accompanied with attenuation of interstitial tissue including nerve fibres.[7,26] The number of chief cells is increased with a concomitant increase of sustentacular cells as well. An increased number of mitotic chief cells has been found in hypoxemic rat carotid bodies.[27] Some studies report an increase in the differential count of the dark variant of chief cells and depletion of naturally fluorescent biogenic amines.[13,23]

Carotid body hyperplasia has been documented in individuals dwelling at high altitude compared to individuals living at sea level.[23,28,29] Additionally, hyperplasia may occur under normobaric conditions in individuals with pulmonary disease, cyanotic heart disease or systemic hypertension with ventricular hypertrophy.[8,30,31] The observed hyperplasia of the carotid body in the situations mentioned above, primarily appears to be a physiologic consequence of sustained chronic hypoxia.[2,17] Similarly, hyperplasia of vagal and aorticopulmonary bodies have also been described, suggesting that these paraganglia posses chemoreceptive properties as well.[32]

Of great interest are the reports that describe an increased prevalence of carotid body tumours among populations that dwell at high altitudes.[28,33-35] Although a genetic predisposition for carotid body size or tumour formation among high altitude dwellings can not fully be excluded, the increased prevalence suggest that chronic hypoxia also may present a risk factor for the induction of carotid body tumours. This development may possibly occur via progression from hyperplasia as has been documented in the development of adrenal pheochromocytomas.[17,28,35]

Despite some case reports of paragangliomas in humans with cardiopulmonary disease,[36] there is no convincing evidence that these conditions present a risk factor for development of paragangliomas under normobaric conditons.[17,37,38]

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2. Head and Neck Paragangliomas

History

In the eighteen century, Haller (1743) provided the first anatomical description of the carotid body (ganglion minutum).[39] A detailed histological study of the carotid body-paraganglion was published by Lushka in 1862.[40] Almost thirty years later, Marchand published the first scientific report on carotid body tumours (1891) in which he referred to an unsuccessful extirpation of a carotid body tumour by Riegner in 1888.[41] The patient did not survive. The first successful resection of a carotid body tumour was reported by Albert in 1889, however severe cerebrovascular complications during resections were common until the later decades of the twentieth century.

In 1935, a vagal body tumour arising from the nodose ganglion of the vagal nerve was described by Stout, but remained unnoticed until Lattes re-presented this neoplastic entity in 1950.[42,43]

In an anatomical study of 88 temporal bones (1941), Guild discovered paraganglionic tissue at various locations and identified the jugular and tympanic paraganglion (commonly referred to as the glomus jugulare and glomus tympanicum).[44] Four years later, Rosenwasser reported the removal of a tumour from the middle ear, which was identified as a glomus jugulare tumour.[45] Retrospectively an earlier report of the Dutch pathologist Lubbers in 1937 of a middle ear tumour was also appreciated as a paraganglioma and subsequent publications of this new neoplasm of the middle ear followed quickly.[46]

As mentioned before, paragangliomas have been reported at various other locations in the head and neck region such as larynx, trachea, orbit and the nose.[6,9,47]

Incidence

Head and neck paragangliomas (**HNP**) are rare neoplasms. Various authors have estimated the clinical incidence of HNP between 1/10.000 and 1/100.000.[48,49] However these estimates are probably lower than the necropsy incidence-rates due to often asymptomatic and clinically favourable nature of these tumours.[50] Incidence may also vary due to clustering of hereditary patients as has been demonstrated in the Netherlands or higher frequencies of HNP among high altitude dwellers.[34,51,52] Several studies have reported a female predominance, especially

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in series of carotid body tumours among high altitude dwellers.[33-35,53] Possibly, differences in the development of chemoreceptive-reflexes between males and females may contribute to higher tumour incidences among females, particularly at high altitudes.[53]

Clinical presentation

HNP have been recorded from early childhood to old ages.[54] The average age at time of diagnosis varies in studies between 35 and 55 years.[49,55] Generally, HNP have a slow growth rate, a characteristic that is reflected in a considerable delay between the first symptoms and establishment of the diagnosis, which averages between 4 and 7 years.[49,56] Presenting symptoms vary with the location and extent of the tumour. Common (preoperative) symptoms are a slow growing neck mass or bulging of the oropharyngeal wall, a pulsatile thrill, bruit or tinnitus, and cranial nerve dysfunction.[54-57] Jugulo-tympanic tumours may manifest with skull base invasion and intracranial tumour extension.[57] Especially in hereditary cases, tumours may develop at multiple locations including pheochromocytomas (multicentricity).[58]

Histopathology

Microscopic findings

Characteristically, HNP are arranged in Zellballen that resemble the organisation of the original paraganglion. The Zellballen are embedded in a vascular stroma and demarcated by a fibrous pseudocapsule. (Figure 1.7) Central necrosis or fibrous septa may be present. Extensive fibrosis may cause displacement and distortion of tumour nests with loss of the characteristic architecture. Occasionally, remnants of the original paraganglion may be present at the periphery of the tumor.[17] Generally, there are no features of chronic inflammation in tumour or surrounding tissues and infiltration with inflammatory cells is rarely observed. Le Compte recognized three histopathological patterns: usual, adenoma and angioma-like.[59] These patterns are merely descriptive and constitute no clear clinical difference although some authors have reported that the adenoma-like pattern may display a less favourable biologic behaviour.[60]

HNPs appear to derive mainly from the chief cell component and usually have a higher density of chief cells than their non-neoplastic counterparts in normal paraganglia. The neoplastic chief cells are usually ovoid with greater cellular and

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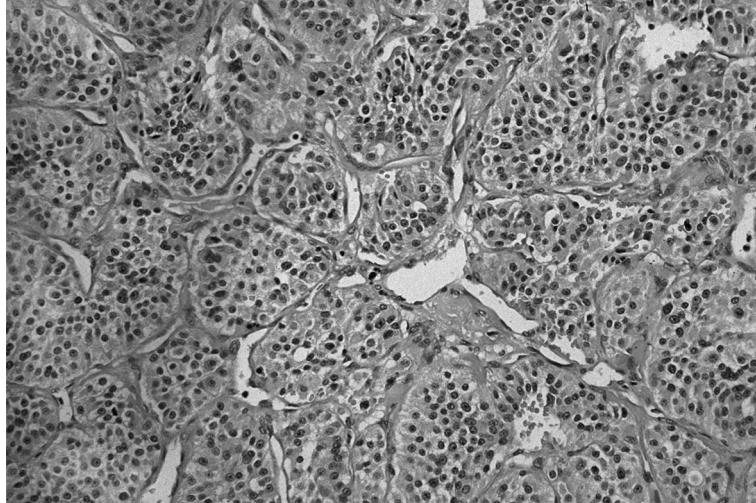
Figure 1.7: Head and Neck Paraganglioma, typical *Zellballen* architecture (HE 25x; colour image on page 139).

nuclear pleomorphism. There is often marked nuclear hyperchromasia but mitotic figures are rarely observed.

There is some controversy concerning the existence of sustentacular cells in HNP. With the use of S-100 immunohistochemistry their presence has been demonstrated in the majority of HNP although with decreased numbers.[61] Some authors have reported that a reduction of sustentacular cells is associated with poor clinical or malignant behaviour.[62] The nature of sustentacular cells in paragangliomas is still a matter of debate. Their presence, albeit with reduced percentages, appears to conflict with a monoclonal expansion of neoplastic chief cells. To explain this controversy, several authors have suggested that sustentacular cells may constitute an additional population of neoplastic cells derived from the same lineage as the chief cells, and HNP should therefore be considered biphasic tumors.[18,61,62] On the other hand, sustentacular cells could represent a reactive stromal component, which is induced by the neoplastic chief cells.[63]

Ultrastructural findings
HNP have been studied extensively with electron microscopy (EM). Although presently its clinical and diagnostic value is limited, EM has yielded some important characteristics of paraganglia and tumours. A constant and significant feature of all paraganglionic chief cells is the presence of neurosecretory granules in the cytoplasm. This finding strongly advocates a neuroendocrine lineage. Another

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important observation is the abundance of mitochondria that often create an oncocytic appearance of the chief cells. The basal lamina between chief cells and capillaries is usually incomplete with chief cells extending directly in the vascular spaces. Other ultrastructural studies describe the loss of architecture and organisation of the chemoreceptive unit with loss of small nerve endings and decrease or absence of sustentacular cells. Finally, most studies describe an increase of chief cells over all other cellular components, indicative of a neoplastic nature of paragangliomas.[17,18,64-69]

Immunohistochemistry

Immunohistochemical studies on HNP have resulted in a generally accepted immunohistochemical profile that is commonly applied in the establishment of the histopathological diagnosis. The chief cells show immunoreactivity for various antigens of which Neuron-specific enolase, Chromogranine A and Synaptophysin yield reliable staining results. Sustentacular cells can be accurately identified with S-100 or Glial fibrillary acidic protein.[14] The introduction of proliferation markers such as PCNA and Ki-67 have created new tools to determine the fraction of tumor cells that is actively progressing through the cell cycle and cell division, resulting in a proliferation index.[70] Although results varied between studies depending on the methods used and the tumour-populations studied, benign HNP generally showed modest or low proliferation indexes with the majority of indexes being smaller than 5%.[71-74] Several studies on pheochromocytomas have reported correlations between the proliferation-indices and clinical behaviour with higher indices in malignant cases. [75-77] In a similar study on HNP using KI-67, only 20% of the tumours proved to be positive and scores did not correlate with clinical parameters.[71]

Flow cytometry

Several flow cytometric studies have demonstrated that a substantial fraction of HNP are DNA-aneuploid, indicating the presence of numerical and probably also structural chromosomal aberrations. Interestingly, also several studies report a considerable number of tumours with tetraploid (sub)populations or elevated G2/M fractions. These findings further support the neoplastic nature of HNP. Although some authors report that DNA-aneuploid tumours tend to behave more 'aggressive', no strong correlation between aberrant DNA-content and an unfavourable clinical course could be established for HNP.[60,78-80]

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Growth rate

HNP are characterised by a slow growth-rate. In a study by van der Mey *et al.,* long term follow-up of a series of patients with HNP revealed no clinical progression of untreated tumours over a period of 15 years.[81] In a recent study, radiological growth was observed in only 60% of tumours examined. The tumour doubling times in that subpopulation varied between 0.6 and 21.5 years with a median tumour doubling time of 4.2 years.[82]

Due to this indolent growth rate, the natural course of the disease is generally favourable and patients may benefit from a conservative approach rather than surgical treatment with associated morbidity, especially in cases of vagal nerve tumours or a jugulotympanic localisation.[81,83]

Malignancy

Although uncommon, malignant paragangliomas have been reported with varying frequencies. Malignant HNP have been reported from all common tumour sites. Histopathological investigations have not revealed clear criteria that indicate malignant behaviour. Many tumours, both benign and malignant, show nuclear pleomorphism and have aberrant DNA-ploidy-patterns.[78,79] Findings of increased mitotic rate are rare and by itself no proof of malignancy.[14,84] Many tumours show capsular and vascular invasion without presence of metastasis. Jugulotympanic tumours often display erosion of the surrounding bone but this may be a result of the relative tight osseous boundaries at this location rather than true malignant degeneration. As mentioned earlier, immunohistochemical studies with proliferation markers on pheochromocytomas have demonstrated a propensity for higher proliferation indices in malignant cases but similar studies with malignant HNP are lacking.[71,85] There are several studies that have reported a decrease or absence of sustentacular cells in malignant primary tumours and metastases.[14,62] However, the significance of this finding is still a matter of debate because there is no reliable index or number of sustentacular cells formulated that would predict malignant behaviour and S-100 positive metastases have been reported.[79]

Because of lack of unequivocal histological characteristics that delineate malignant tumours from benign cases, malignant tumours are diagnosed on the basis of their clinical behaviour. Presently, HNP are considered malignant if metastasis is

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demonstrated to non-neuroendocrine tissues. The most common metastatic sites include cervical lymph nodes, lung, bone and liver.[85]

The observed frequency of metastasis varies but in most series is estimated at approximately 5%. Among the major tumour sites, jugulotympanic and carotid body tumours have a low propensity for metastasis (4-6%).[86] Vagal nerve tumours as well as sinonasal paragangliomas are reported to have a larger malignant potential with observed metastasis in 19% and 24% respectively.[85] However, these figures may be confounded by various factors such as multicentric disease, small numbers and incorrect histopathological diagnosis. The latter proved to be the case in nearly all malignant laryngeal paragangliomas, which after histological revision could be attributed to other neuroendocrine neoplasm's.[47] A recent large study identified 59 malignant cases among 355.019 HNP diagnosed (0.016%) over a 10-year period.[87] In 68.6% of the cases, metastasis was confined to cervical lymph nodes and 31.4% had distant disease. Most malignant carotid body tumours had disseminated to cervical lymph nodes whereas most distant metastasis could be attributed to HNP from other locations. The 5-year relative survival rate for patients with metastasis was 59.5%. The 5-year survival rate for patients with metastases limited to cervical lymph nodes was significantly higher than that for distant metastases (77% vs 12%). Although survival with metastases is reduced, distant metastases may behave indolent, being relatively harmless to the patient.[88]

Functional activity

Although paraganglion-chief cells have numerous neurosecretory granules that contain catecholamines such as dopamine and norepinephrine, excessive production and release of catecholamines is an uncommon clinical feature of HNP.[89] In a review of the literature, Zak *et al* identified 20 cases of functional HNP and in general the prevalence of vasoactive HNP is estimated at 1%.[2,90] Clinical symptoms of excess catecholamine production include labile hypertension, headache, palpitations and flushing. Although functional HNP are uncommon, all patients should be evaluated for elevated catecholamine production, to avoid catastrophic cardiovascular complications during surgery. Additionally, detection of elevated catecholamine excess may lead to identification of multicentric sympathicoadrenal paragangliomas or pheochromocytomas.[58]

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Association with other conditions

There are numerous reports that describe HNP occurring simultaneously with other neoplasms.[2] Foremost these include sympathicoadrenal paragangliomas and pheochromocytomas as well as other neuroendocrine tumours, neurofibromas and meningiomas. Furthermore HNP have been reported in conjunction with various tumour-syndromes such as Neurofibromatosis type 1, multiple endocrine neoplasia type II, von Hippel Lindau disease (VHL) and Carney's triad (gastric leiomyosarcoma, pulmonary chondroma and functional extra adrenal paragangliomas).[91-95]

Detection and classification

Radiological imaging

Currently, the most sensitive diagnostic radiological technique for establishing the diagnosis of HNP is magnetic resonance imaging. The highly vascularised tumours are greatly enhanced with Gadolinium-contrasts in 3D time of flight MR angiography sequences.[96] Similarly, digital subtraction angiography exploits the highly vascularised nature of HNP and can produce a characteristic blush that is indicative for these tumours. Additionally, this technique results in detailed visualisation of efferent and afferent vessels that supply the tumour and may be utilised for (pre-operative) embolisation and planning.

Although ultrasound is not the best diagnostic technique for the identification of paragangliomas, it is often the initial radiological investigation of an unknown swelling in the head and neck. With additional Colour Doppler imaging techniques intraluminal flow in the vascularised tumour can be demonstrated. Moreover, ultrasound investigation can be combined with fine needle aspiration cytology to narrow the differential diagnosis, especially between HNP and squamous cell carcinomas.[97]

Scintigraphy-techniques with radioactive labelled compounds can be utilised for detection and screening of paragangliomas and other neuroendocrine-tumours. MIBG-scans using I^{123} labelled metaiodobenzylguanidine can detect functional paragangliomas.[98] SMS-scintigraphy that detects somatostatin–receptors and the more recent developed PET-scan utilising different tracers can also be used to detect paragangliomas or other (neuroendocrine) tumors.[99-101]

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Classification

Carotid body tumours are generally classified according to Shamblin *et al,* which reckons with encasement of carotid arteries and involvement of the hypoglossal and the superior laryngeal nerve. [102] Because exact involvement of cranial nerves can only be established peroperatively, the Shamblin classification has some limitations. With the improvement of MR-imaging techniques, tumour size can accurately be determined and correlated with complication rates, offering a preoperative classification tool to predict surgical difficulty.[103]

Jugulotympanic paragangliomas are usually classified according to Fisch in four categories.[104]

Presently there is no uniformly accepted classification for vagal nerve tumours. However all tumours involve the ganglion nodosum of the vagal nerve and other important aspects in tumour description include the relation with the skull base and the possible association with carotid arteries.[83]

Treatment

The usual indolent growth pattern of HNP offers the opportunity for careful contemplation of the most appropriate treatment strategy. Treatment depends on clinical complaints, age at diagnosis, localisation of the tumour and to some extent on presence of multiple paragangliomas.

Generally, extirpation of actively growing carotid body tumours is advocated in young patients. For vagal nerve tumours and the majority of jugulotympanic tumours that are situated in the infra-temporal skull base as well as slow growing cases in the elderly, a conservative 'wait and scan' policy can be adopted. Although these tumours may cause progressive cranial nerve impairment, they usually represent little threat to the patient's survival. Surgical extirpation in such cases is frequently complicated with considerable cranial nerve damage and is often more detrimental than the original disease.[81,83,88]

Surgical therapy may be motivated by the prevention of metastasis, but HNP should be considered a benign condition with low risks for metastatic dissemination. Even if distant metastases have developed they often have a similar indolent nature as the primary tumour and cause little harm to the patient. Therefore some authors state that the aim of therapy should be to reduce morbidity for the patient rather than to focus upon extirpation of the tumour.[88]

Besides surgical intervention, radiotherapy can be used in selected cases. Although

some studies report good results of radiotherapy for HNP,[105,106] its application is heavily debated. Several authors believe that control and tumour-regression after radiotherapy are related to the natural course and indolent growth patterns of HNP.[107] In a histopathological study Hawthorne demonstrated that tumour behaviour can be unpredictable after radiotherapy and he concluded that radiation should be reserved for the elderly and those in poor health with the aim of retarding local tumour growth.[108] The recent introduction of Gamma Knife and stereotactic radiotherapy for the treatment of HNP yields comparable results as conventional radiation but lacks long term follow up, which is essential in these patients.[109-111]

3. Genetics & Hereditary aspects

History & Linkage mapping

Various early reports on hereditary patterns in the occurrence of HNP have been published. In 1980, van Baars in his thesis reported an age dependant autosomal dominant pattern of inheritance in several affected families with HNP.[112] In 1989 van der Mey *et al.,* described an apparent sex specific transmission of the disease. Only children of male carriers (affected or not) develop tumours whereas the offspring of female carriers remains unaffected.[113] This mode of inheritance suggested the existence of a maternal genomic imprint of the disease. Linkage studies on large Dutch families led to the mapping of two putative loci: PGL1 on chromosome 11q23 and PGL2 on chromosome 11q13.[114-116] Differential loss of chromosome 11q in hereditary and sporadic HNP was subsequently confirmed with other techniques such as Comparative Genomic Hybridisation (CGH) and DNA-microsatellite analysis.[117,118]

In 2000, succinate dehydrogenase subunit D (SDHD) of complex II of the mitochondrial respiratory chain (see next section) was identified as the susceptibility gene for PGL1 using a positional-candidate strategy.[119] Subsequently mutations in the genes of two other subunits of complex II were identified in hereditary paragangliomas by a direct candidate gene approach. Germline mutations in SDHC (chromosome 1q21;PGL3) were discovered in a large German pedigree with HNP and mutations in SDHB (chromosome 1p36; PGL4) were identified in pheochromocytomas and several paragangliomas.[120,121]

Presently numerous studies have confirmed the role of complex II gene-mutations in hereditary and sporadic HNP and pheochromocytomas.[122-125]

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Molecular genetic basis of HNP

The mitochondrial complex II (succinate dehydrogenase (SDH); succinate-ubiquinone oxireductase) is a heterotetrameric protein complex involved in the aerobic electron transport chain and in the tricarboxylic acid cycle (TCA cycle; Krebb's cycle). In the TCA cycle, SDH catalyses the oxidation of succinate to fumarate. The liberated electrons are then transferred to the Q-pool of the electron transport chain through reduction of ubiquinone (Q) to ubiquinol (QH_2).

Complex II comprises four subunits: SDHA, SDHB, SDHC and SDHD and is situated in the inner mitochondrial membrane. The hydrophilic catalytic part of the complex consists of the 70-kDa flavoprotein (Fp, SDHA) and the 30-kDa iron-sulphur protein (Ip, SDHB), which form the SDH-enzyme. The membrane-spanning hydrophobic part of the complex is formed by the 15-kDa SDHC subunit and the 12.5-kDa SDHD subunit that anchor the Fp and Ip in the inner mitochondrial membrane and transfer the generated electrons to the Q-pool. (Figure 1.8)
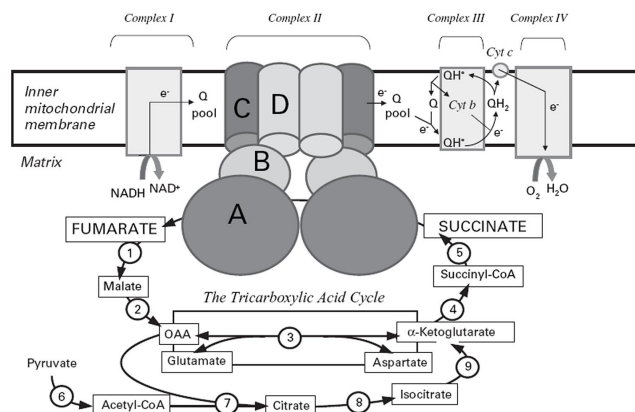
Figure 1.8: Schematic representation of the mitochondrial respiratory chain and the role of complex II. Complex II is composed of four subunits; *SDHA ,SDHB, SDHC* and *SDHD.* The first two subunits form the catalytic domain whereas the latter two subunits anchor the catalytic domain in the inner mitochondrial membrane. Complex II connects the respiratory chain with the tricarboxylic acid (TCA) cycle in the matrix. In the respiratory chain, complexes I and II reduce ubiquinone (Q) to ubiquinol (QH_2). In the TCA cycle, succinate is oxidized by succinate dehydrogenase to generate fumarate. The enzymes involved in this process are: 1) fumarase, 2) malate dehydrogenase, 3) aspartate aminotransferase, 4) a-ketoglutarate dehydrogenase, 5) succinyl CoA synthetase, 6) pyruvate dehydrogenase, 7) citrate synthetase, 8) aconitase, and 9) isocitrate dehydrogenase. (Courtesy of Favier *et al*)[122]

SDH-gene mutations

Numerous studies have described and confirmed the association of gene-mutations in complex-II subunits SDHB, SDHC and SDHD in paragangliomas and pheochromocytomas.[56,125-126] Clinical features that indicate the presence of SDH mutations are primarily the presence of a positive family history, the occurrence of multiple tumours and a low age of onset. Additionally, some studies mention a higher percentage of carotid body tumours in hereditary cases and a higher female to male ratio in sporadic cases compared to hereditary cases (4:1 vs 2:1). However the latter observation may partially be influenced by ascertainment bias because hereditary cases may be concealed after transmission via female carriers. [127,128]

SDHD-gene germline mutations are most commonly associated with HNP.[56,125] Presently 28 distinct SDHD-mutations have been described and the spectrum of mutations continues to expand.[129,130] The majority of the mutations constitute protein-truncating or missense mutations that are predicted to cause loss of function or substantial reduction in SDH-function due to disassembly of complex II.[131] Recently, whole-gene deletions of SDHD and partial deletions of SDHB and SDHC have been described.[132,133] In the Netherlands nearly all HNP with a positive family history are caused by three SDHD founder mutations (D92Y, L95P and L139P). Additionally, genetic analysis of sporadic HNP revealed SDH germline mutations in approximately 30 % of cases examined, the majority of these concerning SDHD-mutations.[130,134] Thus far there is no evidence of somatic SDH-mutations being involved in tumourigenesis of paragangliomas with the exception of a single reported case of a somatic SDHD mutation in a pheochromocytoma.[135] Patients with SDHD mutations often develop multiple tumours that are generally clinically benign. SDHD mutations were detected in apparent sporadic pheochromocytomas as well.[136] However, the prevalence of pheochromocytomas among SDHD-linked HNP-patients has remained unclear.[56,137-139] Various studies with flow sorted tumour cells have demonstrated loss of the wild type SDHD-allele in paraganglioma with resulting retention of the mutated SDHD-allele. This loss of heterozygosity (LOH) indicates that the SDHD-gene acts as a tumoursuppressor gene in the development of HNP.[124,131]

SDHB-mutations are predominantly associated with the development of (extra)-adrenal pheochromocytomas although simultaneous occurrence of HNP has been reported as well. Carriers of SDHB-mutations have an increased risk for the development of malignant paragangliomas or pheochromocytomas. [56,125,137-140]

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The inheritance pattern is autosomal dominant without parent specific disease transmission as in the case of SDHD-mutations.

SDHC-mutations are rare and have thus far been described in a single German family and several sporadic cases.[120,126,133,141] Like SDHD and SDHB-mutations, LOH was demonstrated in two tumours studied, implying that SDHC also behaves as a tumoursuppressor gene and it is assumed that the mutations in SDHC also result in complete loss of enzymatic activity of complex II.[142]

Genomic imprinting

Thus far all HNP that are caused by an SDHD mutation are transmitted in an autosomal dominant fashion with an apparent imprint through the maternal line.[113,143] However, the observed LOH of the wild type SDHD-allele in tumours conflicts with an imprint of this SDHD-gene copy because in that scenario no selection pressure on the wild type allele is expected.[144] Moreover there is biallelic expression of the SDHD-genes in all non-tumour tissues examined and thus far no molecular signs of actual imprinting of the chromosomal region 11q23 such as methylation could be detected.[119] A new model has been proposed by Hensen *et al*.[145] In their study they have described that in SDHD-linked HNP, there is exclusive loss of the entire maternal chromosome 11. The authors suggest that apart from loss of the wild type SDHD-allele on 11q23, simultaneous loss of a second maternally imprinted tumoursuppressor gene on the distal part of chromosome 11 is required for tumour development to occur. Recently, similar chromosome 11 monosomy was confirmed in an independent study.[146]

4. Models of tumourigenesis of Paragangliomas

Mitochondrial dysfunction-induced tumorigenesis

The TCA-cycle and oxidative phosphorylation are fundamental and vital bio-energetic systems/processes of every cell. It is therefore intriguing how mutations in these cellular systems can result in tumour formation.

Several models have been proposed that address the transition from disturbed mitochondrial function to neoplastic growth after mutations in SDH-subunits. These include decreased apoptosis or programmed cell death, enhanced production of reactive oxygen species (ROS) and the generation of an erroneous hypoxic signal under normoxic conditions (pseudo-hypoxia). It is conceivable that all three major

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models may act jointly and promote tumourigenesis in HNP and pheochromocytomas.[124,147]

Apoptosis

Programmed cell death is an important cellular mechanism to eliminate cells that have acquired properties, which could result in dangerous autonomous growth. The capability of tumour cells to circumvent apoptosis is one of the hallmarks of neoplastic growth.[148,149]

Mitochondria harbour important proteins from which apoptotic pathways are activated and therefore can play a prominent role in orchestrating apoptotic processes.[150,151] Likewise it is conceivable that chronic mitochondrial dysfunction could hamper or attenuate apoptosis and thereby contribute to tumour development. Several *in vitro* studies indicate that SDH may function as a modulator of apoptosis. Transient reduction of SDH-activity was found to promote apoptosis whereas SDH-deficient Chinese Ovary Hamster cells are defective in their apoptotic response to several stimuli.[152] In a second study, prolonged culture of SDH-deficient mouse fibroblasts resulted in tumourigenic growth.[153] Another study described that downregulation of SDHD expression protected pheochromocytoma cells from apoptosis after Nerve growth factor (NGF)-withdrawal.[154]

Besides these possible modulatory effects of SDH-activity on apoptosis, other anti-apoptotic mechanisms could be present in chief cells or may have been acquired during tumour progression and will be discussed later in this chapter.

Oxidative stress and ROS-formation

Complex II is involved in the oxidation of succinate to fumarate in the TCA-cycle. The free electrons that are generated during this process are subsequently transferred to ubiquinone and further reduced in the electric transport chain. Several studies have suggested that during oxidative stress or hypoxia, complex II may generate free radicals that subsequently lead to the production of ROS.[155,156] ROS could function as part of a mitochondrial oxygen sensor and serve as a downstream signal during oxidative stress or hypoxia and lead to carotid body discharge and hyperventilation within seconds.[21] Similarly, ROS could also activate hypoxic stimulation pathways such as stabilisation of the transcription factor Hypoxic Inducible Factor 1a (HIF).[157,158]

Because the SDH-gene mutations in HNP and pheochromocytomas are predicted to cause loss of function of the encoded subunits and consequently reduce complex

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II activity, it is conceivable that this may lead to aberrant generation of ROS and subsequently result in constitutive activation of hypoxia pathways such as HIF. This could eventually lead to a proliferative response and tumourigenesis.[122] Additionally, ROS formation may induce oxidative damage to DNA and thus leading to nuclear hypermutability and tumour development.[153,159]

Although both mechanisms present interesting models for tumourigenesis in HNP, structural analysis of bacterial SDH revealed that it is not a major site for ROS generation but probably serves as an electron sink for ROS that is generated distal from the Fp site.[124,160] Additionally, elevated ROS formation also resulted in enhanced apoptosis and actual transformation of cultured cells occurred only after prolonged times.[124,161]

Pseudo-hypoxia

The carotid body and (extra) adrenal paraganglia are hypoxia-responsive organs that are involved in oxygen sensing in adult life and during foetal development. Because carotid body tumours are the most common type of HNP and the prevalence of carotid body tumours is increased at higher altitudes, there has been a long standing hypothesis that chronic hypoxia or disturbances in oxygen sensing could be involved in the tumorigenesis of HNP.[33] The recent demonstration that environmental oxygen pressure exerts a modifying effect on the penetrance and expressivity of SDHD-linked HNP has led to further support of this hypothesis and suggests that SDH plays an important role in cellular oxygen sensing in paraganglia.[143,162] Accordingly, perturbation of SDH-activity could generate an erroneous hypoxic signal under normoxic conditions that could eventually initiate a proliferative response: *pseudo-hypoxia*.[123,131,163]

Experimental data with a heterozygous SDHD-knockout mouse-model showed that the mutation leads to partial deficiency of SDH-activity and persistent enhancement of resting carotid body-activity.[164] Moreover the affected carotid bodies showed subtle hypertrophy and hyperplasia, phenomena that also have been observed in carotid bodies from high altitude dwellings in the Andes-mountains and could precede neoplastic transformation.[23,28] Recent studies indicate that SDH-dysfunction leads to stabilisation of HIF and activation of hypoxia pathways such as the expression of Vascular Endothelial Growth Factor (VEGF) in SDHD and SDHB-linked paragangliomas and pheochromocytomas.[165,166] HIF is an important transcription factor that is stabilised under hypoxia after which it translocates to the nucleus where several target genes are transcribed that are involved in

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proliferative pathways and angiogenesis.[167,168] The stabilisation of HIF is controlled by the Von Hippel-Lindau protein (pVHL), named after the VHL-syndrome in which this protein is defective due to mutations.[169,170] VHL leads to a varied spectrum of highly vascularised tumours among which pheochromocytomas and sporadically paragangliomas occur.[171-173] Interestingly, a recent microarray study on classification of pheochromocytomas demonstrated a clustering of VHL and SDHB-derived tumours based on HIF-responsive genes, suggesting that a common HIF-controlled pathway is involved in the tumourigenesis of both of these inherited tumour-syndromes.[174] A possible mechanism that links SDH-dysfunction with HIF-stabilisation has recently been described by Selak *et al.* As SDH-activity is reduced, succinate will accumulate and lead to competitive inhibition of specific prolyl hydroxylases that alter the degradation susceptibility of HIF.[175] Additionally, it has been demonstrated that inhibition of prolyl hydroxylase activity also attenuates apoptosis after NGF withdrawal in pheochromocytomas and could thus provide an alternative factor that contributes in the development of paraganglionic neoplasms.[154]

Although these experimental data indicate an important role for HIF related pathways in SDH-linked tumours and VHL, these models cannot fully explain the tumourigenesis of all pheochromocytomas because in some VHL-cases, pVHL still targets HIF for degradation. Apparently pseudo-hypoxia can also initiate alternative pathways, independent from HIF and its transcription targets.[124]

One possible candidate is the angiogenic basic fibroblastic growth factor (bFGF). Immunohistochemical studies have demonstrated the simultaneous presence of bFGF and its high affinity receptor FGFR1 in carotid bodies and pheochromocytomas and experiments on cultured carotid body chief cells indicates that basic fibroblast growth factor (bFGF) may act as a survival factor under hypoxic conditions, capable of inducing proliferation.[176-178]

Proliferation, Cell Cycle control and Apoptosis

Apart from the role of mitochondrial tumoursuppressor genes and their association with oxygen sensing in the development of HNP, the natural behaviour constitutes another intriguing aspect of these neural crest derived tumours. An outstanding clinical hallmark of HNP is their apparent indolent behaviour. As noted before, the average tumour doubling time is extremely long compared to other tumours and is an important factor in treatment strategies.[81,82,90]

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After the induction of a mitogenic signal the actual growth of a tumour is determined by various factors such as proliferation and apoptosis as well as circumstantial processes such as angiogenesis and interactions with surrounding matrix and tissues.[148]

Cell Cycle control and proliferation
In order to proliferate, the number of tumour cells needs to increase through mitosis. Under normal conditions mitosis is tightly governed through a process of successive steps that the dividing cells have to proceed before actual division can be realised: the *Cell Cycle*. Each step or level in the cell cycle is driven by different factors and the proper completion of processes and preparations is monitored or checked before proceeding to the next step in the cell cycle through molecular checkpoints.[179,180] Failure or irregularities can lead to an arrest in the cell cycle and eventually initiate apoptosis.[181,182] This latter process serves as the ultimate safeguard to prevent fatal errors in the mitotic process that may lead to improper function of cells and autonomous growth.[149,183]

Neoplastic growth requires a constituvely activated mitogenic signal that continuously drives the cell cycle. Secondly, most tumours must acquire properties to circumvent checkpoints in order to proliferate in an autonomous fashion. All of these properties have been found at various levels of the cell cycle in many neoplasm's, often combined with apoptotic resistance.[148,184]

As mentioned before, HNP have generally maintained a histological organisation that appears similar to the architecture of the original paraganglion including the presence of sustentacular cells.[17] Although nuclear pleomorphism in chief cells can often be observed, mitoses are rarely present and Ki-67 proliferation indexes are low.[71,74] On the other hand, flow cytometric studies have demonstrated DNA aneuploidy and tetraploidy in considerable fractions of tumours examined (~25%-50%) indicating the presence of numerical and probably also structural chromosomal aberrations.[60,78,79,185,186] Even though the latter findings strongly suggests that HNP should be considered true neoplasm's, the number of proliferating tumour cells is remarkably low and apparently there is no strong propensity for malignant degeneration. This could imply that in HNP the mitogenic stimulus is weak or that several cell cycle checkpoints are still operational. This observation is supported by a limited number of studies reporting that inactivation of the cell cycle-protein p53 is not implicated in the development of HNP.[185,187,188] p53 is a crucial protein in cell cycle regulation and is involved in the activation of

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several checkpoints and initiation of repair or apoptosis. For these reasons, p53 is frequently inactivated in many tumours due to mutations or inhibition of downstream pathways.[182]

Apoptosis

As has been pointed out earlier, several lines of evidence also indicate that SDH dysfunction may result in abrogated apoptosis and subsequently contribute to development of HNP. Other anti-apoptotic mechanisms may be involved in the tumourigenesis as well. At the mitochondrial level, apoptosis is controlled by several stimulatory and inhibitory proteins that belong to the Bcl-family.[189] The inhibitory proteins Bcl-x$_L$ and Bcl-2 are widely distributed in neural tissues and prevent apoptosis by blocking the release of mitochondrial cytochrome-c, thus preventing the activation of pro-apoptotic caspase pathways.[151,190-192] Therefore, overexpression of inhibitory Bcl-proteins is a potential mechanism in tumourigenesis and has been reported in several neoplasm's including paragangliomas.[149,193-196] Several reports indicate that these proteins are also able to prevent hypoxia-induced cell death and stabilise mitochondrial membrane potentials, thus contributing to mitochondrial homeostasis. Possibly mitochondrial dysfunction due to SDH-mutations, could lead to elevated expression of Bcl proteins in chief cells and consequently to abrogation of apoptosis.[197-199]

Micro-environmental conditions

Angiogenesis

In order for tumours to expand, angiogenesis is important for the supply of nutrients and oxygen. As mentioned previously, HNP and pheochromocytomas are highly vascularised tumours with ample blood supply. Probably, the hypervascularity is a result of the association of the normal precursors of the tumour cells with oxygen sensing and the subsequent conditions of pseudo-hypoxia, leading to the activation of hypoxic pathways such as expression of VEGF, that stimulate angiogenesis as explained previously.[166,200-202]

Immunosurveillance

It has been postulated that due to their changed antigenic constitution, tumour cells can provoke a cellular immune response of various extent. In many tumours, an immune reaction is present with infiltration of leucocytes and areas of necrosis.

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Therefore, evading strong cellular immune reactions may be important in the development or progression of neoplastic growth.

No obvious immune response has been observed in HNP. The absence of significant infiltration of leucocytes is a common characteristic in paragangliomas and necrosis is only rarely observed.[17] Possibly due to the indolent behaviour of the tumour or because of a low immunogenicity of chromaffin cells in general, immuno-surveillance does not appear to play a major role in the development of HNP.

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5. Aim of the thesis

The purpose of this clinicopathological study is to gain more insight in the molecular-biological processes involved in the development of HNP. Because HNP frequently display an indolent behaviour and grow slowly, the natural course of these tumours plays an important role in treatment strategies for the clinician. In the light of complicated and debilitating surgical procedures, a conservative approach is often a serious alternative in the treatment of HNP. Further understanding of the natural course of these tumours and possible identification clinicopathological parameters that predict the behaviour of these tumours could aid the clinician in his decisions.

The recent identification of SDHB, SDHC and SDHD as susceptibility-genes in heritable paragangliomas and pheochromocytomas has revealed major new insights in the role of mitochondrial tumoursuppressor genes in the development of paragangliomas. The relation of SDH with cellular oxygen sensing and the role of SDH-dysfunction with hypoxia pathways are presently further explored. The phenotypic consequences of SDHD-mutations in HNP are largely unknown and one of the major questions that is addressed, is how SDHD-mutations affect the structure and activity of the SDH enzyme complex. Therefore in **chapter 2**, an immuno-histochemical and enzyme-histochemical study was performed to determine the presence or expression of the two catalytic subunits of complex II as well as SDH-activity in HNP. Additionally immunoelectronmicroscopy was performed to study the morphology of mitochondria and the localisation of complex II subunits in the chief cells.

Recent studies have shown that SDH-mutations, lead to stabilisation of HIF and subsequently to activation of HIF-regulated hypoxia pathways in head and neck paragangliomas and pheochromocytomas. These pathways probably generate a proliferative stimulus leading to tumour development. However, there is insufficient evidence that HIF-pathways can support tumour development by itself and probably alternative pathways are also involved in tumour development. Earlier studies have reported on the possible role of the angiogenic growth factor bFGF in carotid bodies and pheochromocytomas. Because this growth factor showed mitogenic properties and contributed to chief cell survival in these studies, we investigated the immunohistochemical expression of bFGF and its high affinity receptor FGFR1

in HNP and carotid bodies in **chapter 3**.

An intriguing characteristic of HNP is their indolent behaviour and slow growth. Apart from the exact nature of mitogenic stimulus that is generated, further cellular events in the development of these tumours are poorly understood. In **chapter 4**, the proliferation index of HNP is determined and various cell cycle markers, presence of apoptosis and anti-apoptotic markers are investigated. In conjunction with flow cytometric data, a model is constructed that could explain the molecular events that result in the slow and indolent growth of HNP.

The presence of two distinct cell types has always produced controversies concerning the neoplastic characteristics of paragangliomas. Although generally paragangliomas are considered as neoplastic proliferation of chief cells, the nature of sustentacular cells has remained obscure. Some authors consider the sustentacular cells as an alternative differentiation of a single neoplastic cell type and regard HNP as a biphasic tumor. Other investigators consider sustentacular cells a stromal component that is induced by the neoplastic chief cells. In **chapter 5** the nature of sustentacular cells is determined with multi-parameter flow cytometry and heterozygosity-analysis of the SDHD-gene on sorted cell populations.

In **chapter 6** the phenotypic dichotomy of SDHD-mutations is investigated. In a clinical study, initiated by the Dept. of Endocrinology, the simultaneous presence of catecholamine-producing pheochromocytomas among patients with SDHD-linked HNP is determined. To confirm the role of SDHD as tumoursuppressor gene in these pheochromocytomas, LOH-analysis is performed on suitable tumours.

Finally, in **chapter 7**, the results of the studies reported in this thesis are summarised and a model for the possible tumourigenesis in HNP is further discussed.

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Chapter 2

SDHD mutations in head and neck paragangliomas result in destabilisation of complex II in the mitochondrial respiratory chain with loss of enzymatic activity and abnormal mitochondrial morphology

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Abstract

Hereditary head and neck paragangliomas are tumours associated with the autonomous nervous system. Recently, mutations in genes coding for subunits of mitochondrial complex II, succinate-ubiquinone-oxidoreductase, (SDHB, SDHC and SDHD) have been identified in the majority of hereditary tumours and a number of isolated cases. In addition a fourth locus, PGL2, has been mapped to chromosome 11q13 in an isolated family. In order to characterise phenotypic effects of these mutations, the present study investigated the immunohistochemical expression of the catalytic subunits of complex II (flavoprotein and ironprotein), SDH enzyme activity, and mitochondrial morphology in a series of 22 head and neck paragangliomas. These included 11 SDHD-, one SDHB-, two PGL2-linked tumours and eight sporadic tumours. In the majority of the tumours (~90%) the enzyme-histochemical SDH reaction was negative and immunohistochemistry of catalytic subunits of complex II showed reduced expression of ironprotein and enhanced expression of flavoprotein. Ultrastructural examination revealed elevated numbers of tightly packed mitochondria with abnormal morphology in SDHD-linked and sporadic tumours. Immuno-electron microscopy showed localisation of the flavoprotein on the remnants of the mitochondrial inner membranes whereas virtual no signal for the ironprotein was detected. These results indicate that the function of mitochondrial complex II is compromised in the majority of head and neck paragangliomas.

Introduction

Head and neck paragangliomas are hypervascular tumours that frequently occur in a hereditary context with autosomal dominant transmission and genomic imprinting through the maternal line. Genetic linkage analysis revealed linkage to a locus on chromosome 11q23 (PGL1) and SDHD was subsequently identified as the major susceptibility gene harbouring germ line mutations in PGL1 families with loss of heterozygosity (LOH) for the wild type allele in tumours.[1,2] A second locus has been linked to chromosome 11q13 (PGL2) in a separate family of which the genetic mutation remains to be identified.[3] Recently, mutations were also detected in two genes encoding subunits SDHB and SDHC of complex II in pheochromocytoma and a family with hereditary paragangliomas respectively.[4,5] The mutated genes encode subunits of complex II of the mitochondrial respiratory

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chain, which is involved in the process of oxidative phosphorylation and may also function as a cellular oxygen sensor.[6,7]

Although an increasing number of mutations are being detected in hereditary and sporadic paragangliomas, the phenotypic consequences of these mutations are only slowly emerging.[9] As a consequence of functional loss of subunits of complex II, the enzymatic activity of the remaining complex in vitro is greatly reduced and may lead to destabilisation of the respiratory chain with formation of reactive oxygen species (ROS) that could interfere with cellular oxygen sensing.[10-12] Earlier ultrastructural studies that focused on neurosecretory aspects of paragangliomas mentioned disturbed morphology of mitochondria and recently loss of complex II-activity in pheochromocytomas with SDHB and SDHD mutations has been reported.[13-15]

This study characterises the effects of complex-II mutations on the expression of the catalytic subunits and SDH enzyme activity in tumours with different hereditary background. In a subset of paragangliomas, the ultrastructure of mitochondria was studied as well as the mitochondrial localisation of the two catalytic proteins of complex II.

Material and Methods

Tissue specimens and mutation analysis
Tumour specimens were obtained from the Leiden University Medical Centre, (Leiden, The Netherlands) or the Utrecht Medical Centre (Utrecht, The Netherlands). From all cases the tumour localisation, age at diagnosis, family history and presence of multicentricity were recorded. After informed consent, peripheral blood samples were obtained for DNA isolation and mutation analysis as described previously.[3,16] The PGL2-linked cases were excluded from further mutation analysis because the responsible gene on chromosome 11q13 has not yet been identified. Tumour samples were routinely fixed and embedded, and snap frozen in CO_2-ice cooled isopentane. From several tumours additional tissue was immediately dissected into 1-mm^3 cubes and fixed for routine reflection-contrast and transmission electron microscopy and ultra cryosectioning for immune-electron microscopy.

Normal control tissue was obtained through dissection of the paraganglion at the bifurcation of the carotid arteries of 2 autopsy cases devoid of a clinical history susceptible to influence carotid body function. The bifurcation specimens were retrieved and archived according to the guidelines of the Dutch Federation of

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Medical Research Associations (FEDERA) and fixed in 4% buffered formalin and embedded in paraffin for immunohistochemical analysis.

Immunohistochemistry

Primary antibodies were directed against the 70 kD flavoprotein (mAb-Fp) and 30 kD ironprotein (mAb-Ip) of the catalytic domain of complex II (Molecular Probes, Leiden, The Netherlands). Immunohistochemical procedures were performed as described before with slight modifications.[18] A preincubation step with casein in phosphate buffered saline (PBS) for 20 min was conducted to reduce background staining with mAb-Ip. The primary antibodies were diluted in PBS with 1% bovine serum albumin (PBS/BSA) and applied to the sections overnight in a moist incubation chamber. The next day the sections were processed by the streptavidin-avidin-biotin method (mAb-Fp) or with the Envision® polymer (Dako, Glostrup Denmark) to avoid background staining (mAb-Ip).[19]

Myocardium tissue that contains abundant mitochondria was used as positive control. The immunohistochemical results were evaluated semiquantatively as described before.[20] Briefly, the staining intensity (**0**, no staining; **1**, weak; **2**, moderate; **3**, strong) and the percentage of positive tumour cells (**0**, 0%; **1**, 1% to 24%; **2**, 25% to 49%; **3**, 50% to 74%; **4**, 75% to 100%) were evaluated and both scores were added up to generate a total score. In general, the percentage of positive tumour cells approached 100% in each case. Therefore, a score greater than 5 was considered strongly positive whereas lower scores represent weak staining.

Enzyme-histochemistry

SDH activity was determined with an enzyme-histochemical assay after Pearse with minor modifications.[21] Counterstaining was not routinely performed. Myocardium and kidney tissue were utilised as positive controls. Physiological enzyme-activity in paraganglion tissue was assessed through evaluating activity in tissue-sections of adrenal medulla. The enzyme immunohistochemical results were evaluated qualitatively.

Electron microscopy

Morphological analysis by transmission electron microscopy and immuno-electron microscopy was performed as described before.[22] For immuno-electron microscopy, ultrathin cryo-sections were cut on a Reichert UltracutS/FCS microtome (Leica,

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Vienna, Austria) and sections were collected on carbon-coated collodion films on copper grids. After quenching of aldehyde groups and a preincubation step, the grids were incubated with the mAb-Fp and mAb-Ip in 1 % PBS/BSA. After washing, the grids were then incubated with 10nm gold conjugated goat anti mouse 1:30 diluted (GAM-G10 Amersham) for 2h and further processed as described before.[22]

Results

Clinicopathological findings and mutation analysis

The clinical and pathological characteristics are summarised in Table 2.1. Thirteen patients had a positive family history of which 11 cases were attributed to PGL1 families and SDHD mutation analysis revealed two Dutch founder mutations in this subgroup: the Asp92Tyr mutation in exon 3 and the Leu139Pro mutation in exon 4.[16] Two cases were from an isolated family known to be linked to the PGL2 locus on chromosome 11q13.[3] Nine patients had no apparent family history and were designated as sporadic cases. No SDHD mutations were detected in these cases but subsequent SDHB mutation analysis revealed a splice-donor-site mutation in exon 4 (IVS4+1g>a) in one patient (case 21). One patient (case 19) had a history of bilateral (non-functional) carotid body tumours, meningeoma, a cystic pituitary adenoma and a pleomorphic adenoma of the parotid gland. This constellation of different neoplasms may be related to a tumour syndrome such as Carney's Triad,[23] however, clear evidence was lacking and the family history was unsuspicious.

The age at diagnosis ranged from 19 to 70 years (median 41 years). The average age of hereditary patients was lower than that of sporadic patients (35.3 vs 46.6 years), probably reflecting the generally greater awareness of family members and physicians.

All cases showed a benign clinical course. Microscopically, the majority of tumours had the characteristic organisation of chief cells and sustentacular cells in clusters (Zellballen; Figure 2.1A). One jugulotympanic tumour (No 7) displayed infiltration in adjacent osseous tissue and may be classified as aggressive based on this finding. In one PGL2-tumor (No 13) lymphocytic infiltrates were observed, a rare feature since paragangliomas generally do not elicit an inflammatory response.

Table 2.1 Clinicopathological characteristics, mutation-status, SDH-activity and
immunohistochemical results

tumour	location	fam. history	age	multi-centricity	SDHD mutation	SDHB mutation	SDH-activity	flavo protein	iron protein
1	cbt	PGL1	36	jtt	L139P	-	CC neg	7	3
2	cbt	PGL1	40	vnt	D92Y	-	CC neg	7	5
3	cbt	PGL1	32	cbt	L139P	-	CC neg	6	5
4	cbt	PGL1	49	-	D92Y	-	CC neg	7	5
5	cbt	PGL1	43	-	D92Y	-	CC neg	7	5
6	vnt	PGL1	48	cbt, vnt, pheo	D92Y	-	CC weak	7	6
7	jtt	PGL1	24	pheo	D92Y	-	CC neg	6	0
8	cbt	PGL1	26	-	D92Y	-	CC neg	7	6
9	cbt	PGL1	29	vnt, jtt	D92Y	-	CC weak	7	5
10	cbt	PGL1	40	-	D92Y	-	CC neg	7	4
11	cbt	PGL1	44	vnt, jtt	D92Y	-	CC weak	7	5
12	cbt	PGL2	19	-	-	-	CC neg	5	5
13	cbt	PGL2	29	vnt	-	-	CC neg	6	5
14	cbt	?	33	vnt	neg	neg	CC neg	7	4
15	vnt	spor	42	-	neg	neg	CC neg	6	5
16	cbt	spor	70	-	neg	neg	CC neg	7	5
17	cbt	spor	29	-	neg	neg	CC neg	7	5
18	cbt	spor	43	-	neg	-	CC pos	7	7
19	cbt	spor	51	cbt[*]	neg	neg	CC neg	0	4
20	cbt	spor	49	-	neg	-	CC pos	6	7
21	cbt	spor	56	-	neg	IVS4+1g>a	CC neg	7	5
22	cbt	spor	42	cbt	neg	neg	CC neg	6	5

Table 2.1: cbt: carotid body tumour, jtt: jugulo-tympanic tumour, vnt: vagal nerve tumour, pheo: pheochromocytoma, PGL1: positive family history for PGL1 (locus 11q23), PGL2: positive family history for PGL2 (locus 11q13), spor: sporadic, ?: uncertain family history, D92Y and L139P: SDHD founder mutations, IVS4+1g>a: SDHB mutation, neg: no SDHD or SDHB mutation detected, CC neg, pos, weak: negative, positive or weak staining of chief cells, Immunohistochemical scores for flavoprotein and iron protein are described in the method section, *: other tumours besides carotid body tumours (see text).

Figure 2.1: Routine, immunohistochemical and enzyme-histochemical images of head and neck paragangliomas and control tissue. Inserted scale bars represent 0.05 mm. A: paraganglioma: Zellballen-pattern, B: mAb-Fp staining in myocardium; positive control, C: mAb-Ip staining in myocardium; positive control, D: strong cytoplasmic staining with mAb-Fp in paraganglioma (PGL1), E: weak cytoplasmic staining with mAb-Ip in paraganglioma (PGL1), F: strong cytoplasmic staining with mAb-Ip in paraganglioma (sporadic tumour 20), G & H: SDH-activity in myocardium and kidney tubular epithelium, I: (absent) SDH-activity in chief cells of a paraganglioma (PGL1), insert: HE-staining, J: (positive) SDH-activity in chief cells of a paraganglioma (sporadic tumour # 20) 25x, insert: HE-staining. (Colour image on page 140)

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Immunohistochemistry

The immunohistochemical results are summarised in Table 2.1. Myocardium serving as positive control stained strongly with both mAb-Fp and mAb-Ip (Figures 2.1B and 2.1C). In normal carotid bodies, there was granular staining of chief cells with both the mAb-Fp and mAb-Ip. Chief cells of all but one tumour showed a strong expression of the flavoprotein (median score 7), which generally appeared enhanced when compared to the expression levels in carotid bodies and comparable to expression levels as found in myocardium. In the SDHD- and SDHB- linked cases the expression pattern was diffuse cytoplasmatic (Figure 2.1D), whereas in the PGL2 cases the pattern was more granular (data not shown). The pattern of the sporadic cases varied. Interestingly, in one sporadic tumour (No 19), the expression of flavoprotein was negative in chief cells whereas non-tumour tissue in the same section showed normal staining intensity. In contrast, the iron protein in SDHD-, SDHB-, and PGL2-linked tumours as well as most of the sporadic tumours was generally poorly expressed in the majority of chief cells (median score 5) compared to control tissue and carotid bodies (Figure 2.1E). However, in two sporadic tumours a strong granular pattern was detected in the cytoplasm of the chief cells comparable to expression in control tissue (Figure 2.1F).

Enzyme-histochemistry

Strong SDH-activity was observed in myocardium and renal tubular epithelium (Figures 2.1G and 2.1H). In most SDHD-, SDHB- and PGL2-linked tumours as well as the majority of sporadic tumours, SDH-activity was virtually absent in the chief cells(Figure 2.1I). Occasional lymphocytic infiltrates or stromal components displayed some SDH-activity in the tumours thereby serving as an internal control. However, in two sporadic tumours (Nos 18 and 20) SDH-activity was present in chief cells(Figure 2.1J). These cases with SDH-activity also showed strong immunohistochemical expression of the ironprotein.

Electron microscopy

In two SDHD-linked and one sporadic tumour examined, the chief cells contained abundant numbers of mitochondria, often nearly occupying the complete cytoplasmatic compartment (Figure 2.2A). The mitochondria had a swollen aspect with loss of cristae and often the presence of inclusion bodies (Figure 2.2B). Giant mitochondria were frequently observed (Figure 2.2C). Morphology and numbers

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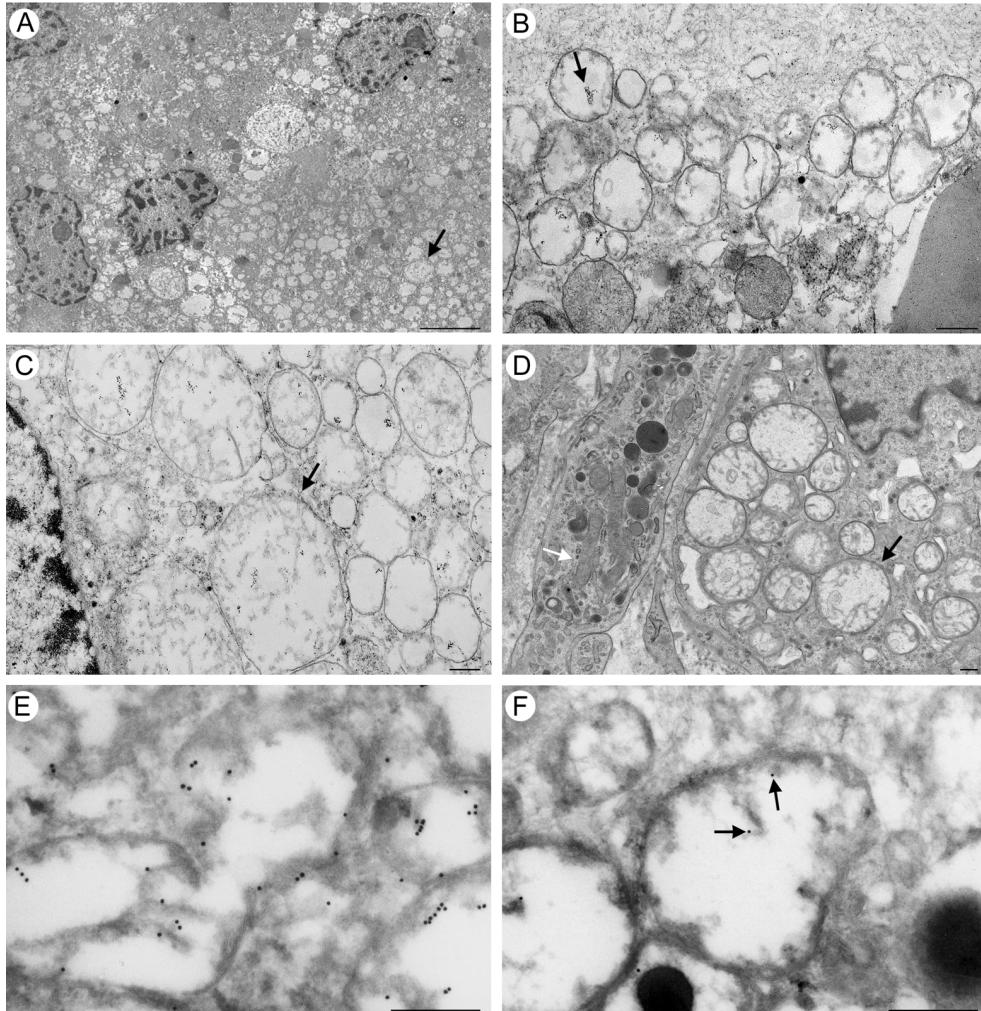
Figure 2.2: Electron microscopic and immunoelectron microscopic images of head and neck paragangliomas. A: Overview with chief cells containing abundant mitochondria (arrow). PGL1-tumour, 2200x, scale bar represents 5 μm. B: Abnormal, swollen mitochondria in chief cells with loss of inner membranes and inclusion bodies (arrow). PGL1-tumour, 5500x, scale bar represents 0.5 μm. C: Giant mitochondria (arrow) in chief cell. PGL1-tumour, 11500x, scale bar represents 0.5 μm. D: Comparison of mitochondrial morphology between chief cell (black arrow) and non-tumour cell (white arrow). Sporadic tumour, 6600x, scale bar represents 5 μm. E: Immuno-electronmicroscopy with mAb-Fp labelling of chief cell; abundant particles in mitochondria. Sporadic tumour, 15500x, scale bar represents 0.5 μm. F: Immuno-electronmicroscopy with mAb-Ip labelling of Chief cell; scarce particles in mitochondria (arrows). Sporadic tumour, 15500x, scale bar represents 0.5 μm.

of mitochondria in several types of non-tumour cells, such as endothelial cells, mast cells and granulocytes appeared normal (Figure 2.2D). Also, in unrelated tissue samples prepared during the same period as the tumours, the mitochondrial morphology appeared normal. The morphological features of the chief cells are similar to those in earlier studies.[13,24]

Chief cells were usually oval or polygonal shaped with ovoid nuclei and besides the numerous mitochondria the cytoplasm was relatively devoid of other organelles.

Immuno-electron microscopy

Incubation of ultrathin cryosections with the mAb-Fp conjugated with gold particles produced strong labelling within mitochondria. The flavoprotein was located on the inner membranes and remnants of cristae and no soluble antigen was detected (figure 2.2E). On the other hand, after application of the conjugated mAb-Ip, virtually no labelled ironprotein could be detected. Occasionally, signal was detected localised on the inner mitochondrial membranes and cristae (Figure 2.2F).

Discussion

Recent studies have established that the genomic defect in head and neck paragangliomas involves mutations in the genes encoding subunits of complex II of the (mitochondrial) respiratory chain.[1,4,5] Mitochondria and in particular the respiratory chain are not only involved in energy transfer but possibly also serve as an intracellular oxygen-sensor due to the close association with cellular respiration.[25,7] As an O_2-chemoreceptor, the carotid body and possibly other paraganglia may have adapted a particular sensitive (mitochondrial) oxygen-sensing system.[26] Mutations in the genes of crucial components of the respiratory chain may disrupt these sensing systems, possibly creating a condition of pseudo-hypoxia through formation of ROS that eventually could result in a mitogenic signal triggering tumourigenesis in paragangliomas.[1] Although an increasing number of mutations are being detected in hereditary and sporadic paragangliomas, the phenotypic consequences of these mutations are only slowly emerging.[9,27]

Complex II is composed of a catalytic domain consisting of a flavoprotein (SDHA) and an ironprotein (SDHB). The SDHC subunit functions together with the SDHD subunit as the membrane anchor of complex II, attaching the catalytic domains to the inner membranes of mitochondria. Associated haem groups are required for

interaction of ubiquinone with the catalytic subunits, and are essential for proper assembly of the entire complex and attachment to the membrane.[6,8,10] Mutations have been described that predict truncated protein formation of subunits as well as missense mutations in critical domains of subunits that interfere with the function of complex II.[27]

In this study loss of SDH activity in chief cells was found in the majority (~90%) of head and neck paragangliomas, including all cases with SDHD-or SDHB-mutations, linkage to the PGL2-locus and the majority of sporadic tumours. Similar loss of complex II activity was recently reported in tissue homogenates of pheochromocytomas with confirmed loss of function SDHD and SDHB alleles.[14,15,28] All SDHD- and PGL2-linked tumours, as well as most of the sporadic tumours showed strong expression of the flavoprotein subunit often to an extent suggestive of overexpression whereas expression of the second catalytic subunit, the ironprotein, was predominantly reduced. Strong signal for the flavoprotein was detected on (the remnants of) the inner membranes whereas signal for the ironprotein was virtually absent. Apparently, the assembly of complex II is disturbed, which results in decreased levels of ironprotein and a simultaneous increase of flavoprotein expression in these cases. SDHB-mutations also impair SDH activity, presumably through inactivation of ironprotein function.[15,28] In one patient a splice-donor- site mutation in exon 4 of the SDHB-gene was detected. The tumour of this patient showed reduced expression of ironprotein and loss of SDH-activity.

Similar results have been reported from in vitro studies where disruption of the SDHC or SDHD gene resulted in decreased levels of iron-protein due to rapid degradation and subnormal levels of flavoprotein bound to membranes. SDH-activity was absent or severely depressed in these mutants[6,10,11] Case reports of patients with localised complex II defects mention reduced activity with overexpression of the flavoprotein.[29]

These findings indicate that perturbation of the expression of catalytic subunits of complex II and impairment of SDH activity are a common feature in the tumourigenesis of the majority of paragangliomas. However normal SDH activity in the chief cells was observed in two sporadic tumours. Interestingly, these two tumours were the only cases where strong expression of the ironprotein was present. Apparently, the catalytic subunits are undisturbed in these tumours resulting in normal SDH-activity. Possibly, the primary defect in these tumours is in a different part of the putative pathway that results in the development of paragangliomas. This could also explain the reported contradictory results of positive SDH-activity in two sporadic tumours.[24]

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Ultrastructural examination of chief cells revealed unusual high numbers of tightly packed mitochondria with a swollen aspect and loss of inner membranes both in SDHD-linked and sporadic tumours. Identical observations were also made in earlier ultrastructural studies on paragangliomas.[13,24] Possibly, the increased number of mitochondria may reflect a compensatory mechanism to maintain sufficient respiratory chain activity and chemoreception after impairment of complex II function.

In conclusion, this study demonstrates that loss of SDH-activity and destabilization of complex II are common features in the majority of hereditary and sporadic head and neck paragangliomas. The downstream pathway that is activated by destabilization of complex II and leads to a mitogenic signal remains to be characterized. Possibly a hypoxia pathway is triggered through activation of hypoxia inducible factor-I (HIF-I) and downstream targets such as vascular endothelial growth factor (VEGF).[14,15,30] In addition destabilization of complex II may directly or indirectly result in decreased sensitivity to apoptosis and thus contribute to cancer predisposition.[27]

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Chapter 3

Basic fibroblast growth factor and fibroblastic growth factor receptor-1 may contribute to head and neck paraganglioma development by an autocrine –or paracrine mechanism.

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Abstract

Paragangliomas are hypervascular tumours arising from neural crest derived paraganglia that are associated with the autonomous nerve system. Mutations in genes coding for subunits of mitochondrial complex II are associated with hereditary paragangliomas and it has been suggested that these mutations result in a pseudo-hypoxic signal triggering tumourigenesis.

Fibroblastic growth factors are hypoxia-inducible angiogenic stimuli that are involved in the angiogenesis and tumourigenesis of several neoplasm's. It has been demonstrated that basic fibroblastic growth factor (bFGF) is a survival factor for cultured chief cells of the carotid body, capable off inducing proliferation.

To examine the role of this growth factor in paragangliomas we studied the immunohistochemical expression of bFGF and its high affinity receptor fibroblastic growth factor receptor 1 (FGFR1) in 7 normal carotid bodies and in 33 head and neck paragangliomas including two malignant cases and their metastases. Immunohistochemical expression of bFGF and FGFR1 in tumours was confirmed by real-time polymerase chain reaction. FGFR1 was moderately present in carotid bodies and there was strong and significantly enhanced cytoplasmatic staining of FGFR1 in all paragangliomas. Chief cells in carotid bodies and tumours showed strong cytoplasmatic staining for bFGF. The results indicate that FGFR1 and bFGF may contribute to the development of head and neck paragangliomas.

Introduction

Head and neck paragangliomas are rare hypervascular tumours characterised by an indolent growth pattern and a strong hereditary context. The tumours originate from the neural crest derived paraganglia, which are associated with the autonomous nervous system and are situated at several locations in the head and neck region. The most prominent site is the carotid body, a chemoreceptive organ in the bifurcation of the carotid artery that is involved in the registration of oxygen levels in peripheral blood.[1]

In hereditary tumours, the succinate-ubiquinon-oxireductase-subunit D (SDHD)-gene on chromosome 11q23 has been identified as the major susceptibility gene. Subsequently also mutations in two other subunits of mitochondrial complex II (SDHB, SDHC) were found to be associated with hereditary paragangliomas and pheochromocytomas.[2] These subunits are important constituents of complex II in the mitochondrial respiratory chain that probably functions as an intracellular

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oxygen sensor. It has been postulated that dysfunction of complex II may disrupt intracellular oxygen sensing pathways and could generate a pseudo-hypoxic signal. Because the chemosensoric chief cells of carotid bodies may have adapted particularly sensitive oxygen sensor systems, such a pseudo-hypoxic signal could eventually promote tumour-formation through activation of hypoxia-pathways and induction of angiogenesis.[3-5]

In previous studies, the role of the angiogenic growth factor bFGF in carotid bodies and chromaffin cells of the adrenal medulla has been investigated in detail. Immunohistochemical studies have demonstrated the simultaneous presence of bFGF and its high affinity receptor FGFR1 in carotid bodies and pheochromo-cytomas.[6-8] bFGF was found to act as a mitogenic factor in these tissues capable of inducing proliferation and increased cell-survival.[9,10] In order to determine if bFGF and FGFR1 are involved in head and neck paragangliomas tumourigenesis, we investigated the expression of bFGF and FGFR1 in sporadic and SDHD-linked tumours as well as in normal carotid bodies. The finding of elevated immunohistochemical expression of FGFR1 in tumours compared with normal carotid bodies, combined with the simultaneous presence of bFGF in tumours, suggests the presence or induction of an auto- or paracrine loop that may contribute to the development of paragangliomas.

Material & Methods

Tissue specimens
Paraffin-embedded tissue samples of 33 head and neck paragangliomas were retrieved from the archives of the Department of Pathology and relevant clinicopathological data were collected including age at diagnosis and clinical behaviour. Tumours were classified as malignant when regional or distant metastasis could be demonstrated. Metastatic tissue samples were also included in the analysis. Peripheral blood samples were obtained from patients after informed consent and SDHD-mutation analysis was performed as described.[11]

Normal control tissue was obtained through dissection of the paraganglion at the bifurcation of the carotid arteries of 7 autopsy cases devoid of a clinical history susceptible to influence carotid body function.[12] The cases selected were age-matched with tumour cases because some studies have reported age-related changes in expression of bFGF and receptors.[13] The carotid bodies were dissected at unregistered intervals but within 24 hours after death of the patient. The

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bifurcation specimens were fixed in 4% buffered formalin and embedded in paraffin for immunohistochemical analysis. All tissue specimens were retrieved and archived according to the ethical guidelines of the Dutch Federation of Medical Research Associations (FEDERA)

Antibodies and controls

Mouse monoclonal antibodies were directed to recombinant bFGF (Transduction laboratories, Lexington, KY) and FGFR1 (kindly provided by Dr. J. Walters, Oxford Brookes University, Oxford, UK). The characteristics of the latter antibody have been described previously.[14] Normal blood vessels and endothelium served as internal positive controls, negative controls were performed by replacing the primary antibody with 1% phosphate buffered saline.

Immunohistochemistry

A three-step indirect immunoperoxidase technique was used on 4 µm thick paraffin sections, mounted on APES pre-coated glass slides with modifications as described.[15] Briefly, after dewaxing and rehydration, the sections were subjected to microwave treatment in a 0.01 M citrate buffer, pH 6.0 in case of the bFGF protocol whereas no antigen retrieval was performed in case of FGFR1. Next sections were incubated for 1 hour with primary antibodies and subsequently with biotinylated rabbit anti-mouse immunoglobulins and biotinylated peroxidase-streptavidin complex (ABC-kit, DAKOCytomation, Glostrup, Denmark). The sections were stained with DAB chromogen during 10 min in case of bFGF and 7 min with DAB combined with imidazole in case of FGFR1. All incubations were performed at room temperature. To facilitate the identification of major cell types in the Zellball-units, serial sections of carotid bodies were routinely stained for Chromogranin-A as well as S-100 and CD31 for identification of chief cells, sustentacular cells and endothelial cells respectively. Assessment of bFGF and FGFR1- expression in carotid bodies and tumours was evaluated semiquantatively as described.[16] Briefly, sections were scored for staining intensity, as related to a positive internal control (**0**, no staining; **1**, weak; **2**, moderate; **3**,strong) and percentage of chief cells stained (**0:** 0%, **1:** 1%-24%, **2:** 25%-49%, **3:** 50%-74%, **4:** 75%-100%). Both scores were added to generate a total score. Two observers scored all sections, reaching consensus in case of differences. The observers were blinded for clinicopathological data of the tumours.

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Quantitative real-time polymerase chain reaction

To confirm immunohistochemical expression of bFGF and FGFR1 in tumours, real-time polymerase chain reaction (PCR) was performed in combination with the Taqman probe technique (Applied Biosystems, Foster City, CA) for both genes as described before.[17] All tumours of which frozen tissues samples were available were included for real time-PCR analysis. The primer and probe sequences for bFGF and FGFR1 were as follows: bFGF-forward: CGACGGCCGAGTTGACGG, bFGF-reverse: CAGGTAACGGTTAGCACACTCC, bFGF-probe: TET-AAGAGCGACCCTCACATCAAGCTACAACTTCA-TAMRA, FGFR1-forward: GAGATGGAGGTGCTTCACTTA, FGFR1-reverse: TACAGGGGCGAGGTCATCA in combination with Sybr Green. Levels of expressed mRNA were normalised as ratios with the expression of the housekeeping-gene GAPDH. A common reference mRNA sample consisting of a mixture of mRNAs isolated from 11 different tumourcell-lines served as positive control.[18]

After measurement samples were electrophoresed on a 2% agarose gel for visual confirmation of the amplification products: a 114 bp fragment for bFGF and a 157 bp fragment in case of FGFR1.[14]

Statistical analysis

Total immunohistochemical scores for bFGF and FGFR1 between carotid bodies and paragangliomas, and between the subsets of SDHD-linked and sporadic tumours were compared with the Student t-test using the SPSS 10 software package.

Results

Clinicopathological characteristics

The average age of the individuals in the carotid body-group and tumour group were 44.6 and 41.2 years, respectively. This difference was not statistically significant (p =0.59). Thirty-one tumour cases were clinically benign. In two carotid body tumours, metastatic spread to a cervical lymph node was present and these cases were classified as malignant. One jugulotympanic tumour showed invasion of surrounding bone and was classified as locally aggressive.

In 21 patients a SDHD germ-line mutation was detected; the D92Y-mutation in 18 patients and the G6825insC, L139P, L95P-mutation in one case each. One patient was linked to a PGL2 family, of which the culprit gene has been mapped to a locus

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on chromosome 11q13 but still remains to be identified.[19] In both malignant cases a SDHD germline mutation was detected. In 11 patients no SDHD germline mutation was found and they were classified as sporadic cases.

Immunohistochemistry

Immunohistochemical scores for carotid bodies and tumours and the results from the statistical analysis are presented in Table 3.1.

FGFR1: Generally the chief cells in the carotid bodies showed weak to moderate cytoplasmatic expression of FGFR1 (average staining intensity: 1.3: Figure 3.1A). The staining of the cytoplasm of the chief cells was sometimes difficult to assess due to retraction and vacuolisation. The average total score for FGFR1 in carotid bodies was 4.4.

27 of 33 tumours stained strongly for FGFR1 (total scores 6-7), generally with all chief cells showing positive immunoreactions (Figure 3.1B). The malignant tumours and the metastatic chief cells had average total scores of 5.5. The average total score for FGFR1 was significantly higher than the average total score in carotid bodies (6.1 vs 4.4, $p < 0.01$). The average total score for FGFR1 between the subsets of sporadic tumours and SDHD-linked tumours did not differ significantly (5.9 vs. 6.1 respectively, $p=0.43$). However, the average staining intensity in the SDHD-linked tumours was slightly but significantly higher than in the sporadic tumours (2.7 vs. 2.2, $p= 0.04$).

bFGF: In each carotid body the chief cells showed extremely strong immunoreactivity for bFGF (average staining intensity: 3, figure 3.1C). Generally, there was also strong cytoplasmatic expression of bFGF in the chief cells of paragangliomas (average staining intensity: 2.5, figure 3.1D). The average staining intensity and average total score in tumours was significantly lower than for carotid bodies ($p= 0.031$ and $p=0.023$, respectively). In some tumours occasional nuclear staining for the ligand was present. In the malignant tumours and in both metastatic samples, the chief cells showed similar strong immunoreactivity for bFGF. The average staining intensity for bFGF in the subsets of SDHD-linked tumours and sporadic tumours was nearly identical and not significantly different.

Quantitative real-time polymerase chain reaction

In all ten tumours analysed, bFGF and FGFR1-mRNA transcripts were detected using quantitative real-time PCR. The average levels for both transcripts are listed in Table 3.1. The levels are expressed as ratios compared to levels of the GAPDH-

FGFR1

	intensity		%	total	e	PCR
CB (7)	**1.3**	**<0.001***	**3.1**	**4.4**	**<0.001***	
PG (33)	**2.5**		**3.6**	**6.1**		**0.5**
sporadic (11)	2.2	0.040*	3.7	5.9		0.47
heriditary (22)	2.7		3.6	6.1		0.52
benign (31)	2.5		3.6	6.2		
malignant (2)	2.5		3	5.5		
metastasis (2)	2		3.5	5.5		

bFGF

	intensity		%	total		PCR
CB (7)	**3**	**0.031***	**4.0**	**7**	**0.023***	
PG (33)	**2.5**		**3.8**	**6.3**		**1.01**
sporadic (11)	2.5		3.6	6.2		0.57
heriditary (22)	2.5		3.8	6.3		1.42
benign (31)	2.5		3.7	6.3		
malignant (2)	2		4	6		
metastasis (2)	2.5		4	6.5		

Table 3.1:Immunohistochemical scores and PCR resultsCB: carotid bodies, PG: paragangliomas, with number of cases in parentheses. Intensity: staining intensity, %: percentage of tissue stained, total: total score, immunohistochemical scoring is explained in the material and methods section. Statistically significant scores are indicated with * for p-values < 0.05 and are specified in the results section.

household gene in the tumours. No reliable mRNA from normal head and neck paraganglion tissue was available to serve as a control, due to post mortem RNA-degradation and the small size of the paraganglionic tissue. Therefore, the mRNA levels only confirm the immunohistochemical expression of bFGF and FGFR1 in the tumours (Figure 3.2). The average RNA-levels of bFGF showed a twofold difference between the subsets of sporadic and hereditary tumours (0.57 versus 1.42, respectively). The observed difference in FGFR1-staining intensity between the subsets of sporadic and SDHD-linked tumours could not be confirmed by real-time PCR.

Discussion

bFGF is a potent angiogenic and differentiation-factor as well as an established mitogen for various tissues. After binding to the membrane-bound high affinity receptor FGFR1, molecular signalling pathways are initiated that may stimulate
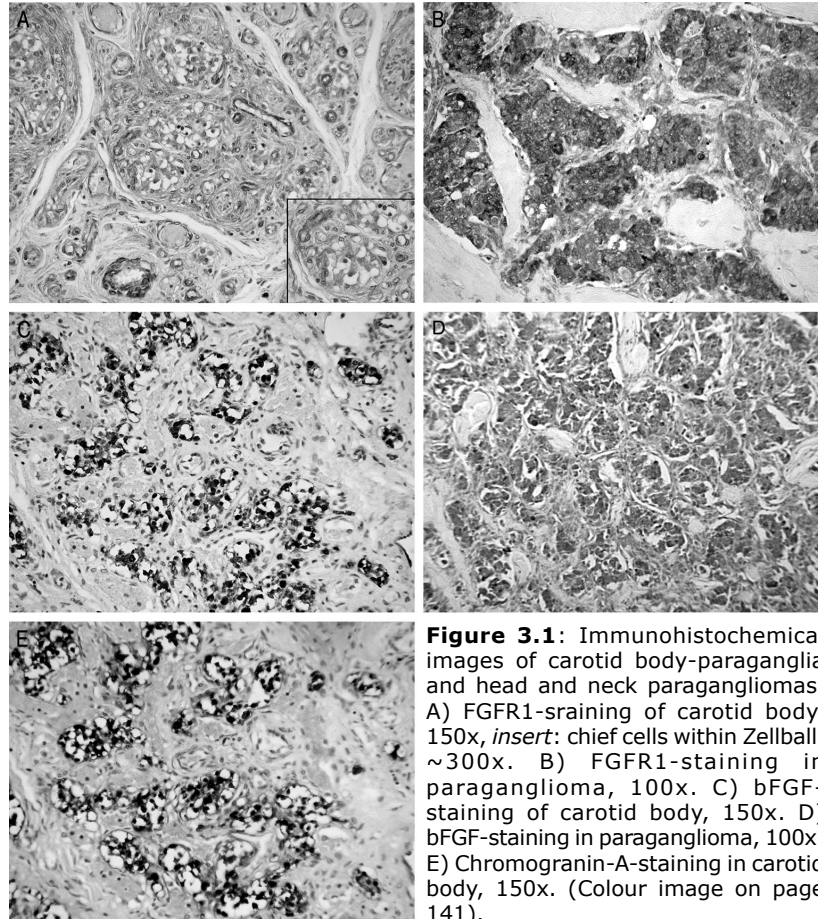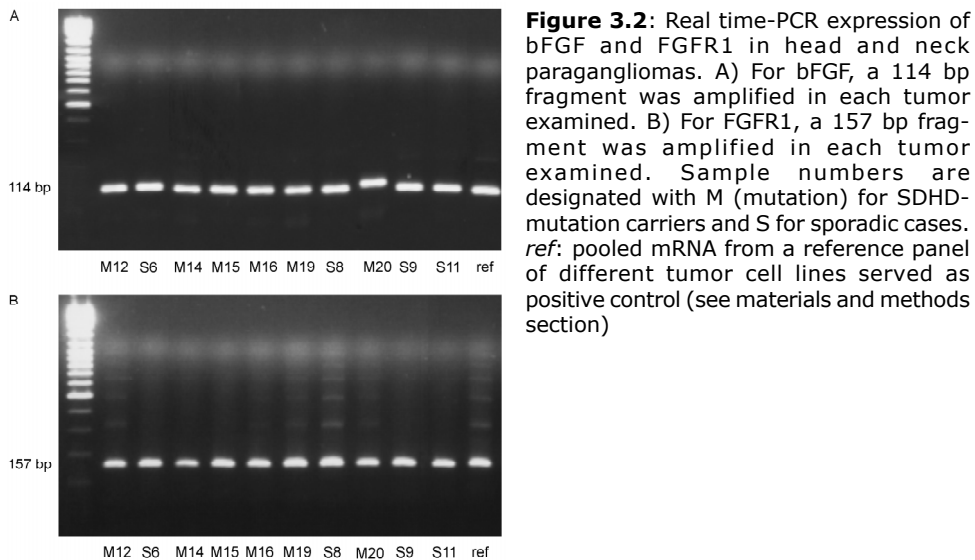
Figure 3.1: Immunohistochemical images of carotid body-paraganglia and head and neck paragangliomas. A) FGFR1-sraining of carotid body, 150x, *insert*: chief cells within Zellball, ~300x. B) FGFR1-staining in paraganglioma, 100x. C) bFGF-staining of carotid body, 150x. D) bFGF-staining in paraganglioma, 100x. E) Chromogranin-A-staining in carotid body, 150x. (Colour image on page 141).

autonomous growth.[20] The growth factor is implicated in the tumourigenesis of several neoplasms including pheochromocytomas.[7,8] In this study we have investigated the expression of FGFR1 and its ligand bFGF in the carotid body-paraganglion and in subsets of SDHD-linked and sporadic head and neck paragangliomas.

Carotid bodies showed moderate cytoplasmatic staining of FGFR1 and strong cytoplasmatic immunoreactivity for bFGF in the chief cells. These findings support the results of previous studies with bFGF on cultured chief cells of carotid bodies as well as experiments on chromaffin cells of the adrenal medulla.[6,7,9]

The majority of paragangliomas showed a strong and significant increase in cytoplasmatic immunoreactivity of FGFR1 in chief cells as compared to age matched normal paraganglia. Also there was simultaneous strong expression of bFGF present

Figure 3.2: Real time-PCR expression of bFGF and FGFR1 in head and neck paragangliomas. A) For bFGF, a 114 bp fragment was amplified in each tumor examined. B) For FGFR1, a 157 bp fragment was amplified in each tumor examined. Sample numbers are designated with M (mutation) for SDHD-mutation carriers and S for sporadic cases. *ref*: pooled mRNA from a reference panel of different tumor cell lines served as positive control (see materials and methods section)

in most of the tumours. Real-time PCR confirmed the expression of FGFR1 and bFGF in a subset of tumours but the lack of mRNA from carotid bodies precluded comparison of the average RNA-levels between normal tissue and tumours. The observed difference in bFGF-mRNA expression between sporadic and hereditary tumours could not be confirmed with immunohistochemistry. This might be explained the semiquantative nature of the immunohistochemical scores as well as possible posttranslational modifications or the presence of external ligand in the tumour cells.

bFGF is a mitogen for chief cells, capable of inducing proliferation and increased cell survival in cultured cells.[9,10] These mitogenic effects of bFGF can be enhanced under hypoxic conditions.[9] In studies on retinal cells and other tissues, a similar enhancement of proliferative responses to bFGF under hypoxic conditions was observed and attributed to upregulation of FGFR1 density.[21,22] Interestingly, in hypoxic retinal cells, a simultaneous reduction of bFGF levels was seen, due to increased intracellular turnover of proteins.[23] This phenomenon may also explain the slightly lower staining scores of bFGF in tumours compared to carotid bodies. Under normal conditions, bFGF-mediated proliferation and cell survival may assist tissues in maintaining homeostasis during hypoxia.[9,21,22] Such a mechanism may have special relevance for the paraganglionic chief cells in the head and neck region. Since these cells are involved in oxygen sensing they may be particularly sensitive to fluctuations in the arterial oxygen concentration. Based on observations

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of increased incidence of carotid body hyperplasia and tumours in dwellings at high altitude and the finding of SDH-dysfunction in hereditary paragangliomas, it has been postulated that head and neck paragangliomas may develop as a result of chronic hypoxic signals. These signals could eventually initiate proliferation and subsequent tumourigenesis in chief cells.[4,24] We hypothesize that under these (pseudo) hypoxic conditions, the chief cells may respond with upregulation of FGFR1. Such an increased expression of FGFR1 could prime chief cells, and result in enhanced (mitogenic) signalling after binding of the ligand bFGF. A similar mode of action was recently found to regulate invasiveness in several hypoxic tumour cell-lines as well as human cervix and breast carcinoma, in which upregulation of the *met* tyrosine-kinase receptor results in increased responsiveness for Hepatocyte growth factor.[25]

The higher staining intensity scores for FGFR1 in the SDHD-linked tumours compared to sporadic cases could reflect chronic impairment of complex II function and subsequent longer duration of pseudo-hypoxic conditions. This could result in more profound expression of FGFR1 in chief cells in hereditary tumours compared to sporadic cases. However the real time PCR results showed no differences in the average mRNA levels between the subsets of SDH-linked and sporadic tumours in support of this hypothesis.

Besides a possible role of bFGF in hypoxic conditions, there are indications that bFGF may also function in the molecular control of the neural crest derived sympathico-adrenal lineage.[26] In the adrenal medulla, bFGF is capable of inducing proliferation of chromaffin chief cells and supports the maintenance of the catechol-synthesizing pathways.[10,27,28] Additionally, it has been demonstrated that in retinal neurones endogenous bFGF is capable of upregulation of Bcl-proteins, which may confer increased resistance to apoptotic insults such as hypoxia.[29] Possibly a similar mechanism may operate in paragangliomas since expression of Bcl-x$_L$ and lack of apoptosis have previously been demonstrated.[30] Other studies on hypoxia-activated pathways have reported the stabilisation of hypoxia inducible factor-1a (HIF-1a) under normoxia and subsequent expression of vascular endothelial growth factor (VEGF) in pheochromocytomas and paragangliomas.[5,31] There are indications that activation of this pathway may interact synergistically with bFGF- regulated pathways by mediating an increase of heparan-sulphate- binding sites on FGFR1 or by inducing enhanced tumour angiogenesis.[32,34] In conclusion, the enhanced expression of FGFR1 and concomitant expression of the ligand bFGF in head and neck paragangliomas may result in a mitogenic signal and possible anti-apoptotic effects that could contribute to the development of these hypervascular tumours.

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Chapter 4

A G2M arrest, blocked apoptosis and low
growth fraction may explain indolent
behaviour of head and neck paragangliomas

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Abstract

Head and neck paragangliomas are characterised by unusually slow growth and a strong hereditary component, which is associated with inactivating mutations in subunits of complex II of the mitochondrial respiratory chain. It is unclear how mutations induce tumourigenesis and lead to the indolent clinical behaviour that often plays a prominent role in treatment strategies. To better understand the natural course of the tumours, we have studied a number of growth-related parameters in 42 hereditary and sporadic paragangliomas. Computerised image analysis showed that the fraction of Ki-67-positive cells was generally below 1%, in accordance with the slow growth. Weak or negative immunohistochemical staining indicated wildtype Tp53 status whereas p-21^{waf} expression was heterogeneous. Most tumours showed strong expression of Bcl-x$_L$ and no apoptotic cells could be detected with the TUNEL assay. Flow cytometry showed abnormal DNA content profiles in 52% of the tumours, including overt aneuploidy as well G$_2$/M arrest or tetraploidization. These results fit into a model in which a stress-activated cell cycle checkpoint at the G$_2$ to M transition and inhibition of apoptosis permit the expansion of only a minor fraction of cycling cells with high likelihood of polyploidisation.

Introduction

Head and neck paragangliomas are rare hypervascular tumours originating from neural crest -derived paraganglia, which are associated with the autonomic nerve system. The carotid body is the major physiologically active paraganglion in the head and neck region, involved in peripheral arterial oxygen registration.

Paragangliomas are usually clinically benign tumours although metastasis has been reported and invasive growth can occur in adjacent tissues.[1] Most tumours, however, are characterised by an indolent growth pattern and often show very slow progression over many years.[2,3]

An important feature is the strong hereditary background of many patients with imprinted transmission through the maternal line and linkage to the PGL1 locus on chromosome 11q23 or PGL2 locus on chromosome 11q13.[4-6] The succinate-ubiquinon-oxireductase-subunit D (SDHD) gene on chromosome 11q23, coding for a subunit of complex II of the mitochondrial respiratory chain, has been identified as the major susceptibility gene in the majority of hereditary paragangliomas (PGL 1) and found to behave like a tumour suppressor gene.[7] Recently, mutations

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were also detected in two genes encoding for subunits SDHB and SDHC of complex II in isolated pheochromocytoma and a separate family with hereditary paragangliomas respectively.[8] Microscopically, the tumours have often retained the histological appearance of the original paraganglion with chief cells organised in typical nests (Zellballen) surrounded by supportive sustentacular cells and embedded in highly vascularised stroma. The presence of nuclear pleomorphism, differences in the ratios of chief cells to sustentacular cells combined with ultrastructural findings such as absence of the supportive elements and the cytometric identification of aneuploid populations, has led to the general agreement that paragangliomas are neoplasms and not merely a manifestation of hyperplasia.[1,9,10]

Because the indolent growth plays an important role in the clinical treatment strategies,[11] studying tumour biological factors that determine the growth rate may increase our understanding of the natural course of these neoplasms and eventually might yield predictive parameters that will aid the clinician in the choice of his treatment strategies.

We studied the expression of the proliferation- associated Ki-67 antigen, cell cycle-related proteins and antiapoptotic proteins in 42 hereditary and sporadic head and neck paragangliomas. Furthermore, we detected the presence of apoptotic cells with the terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end labeling (TUNEL) assay and studied DNA-ploidy by flow cytometry.

On the basis of our data, we propose a model for tumorigenesis of paragangliomas in which an active cell cycle checkpoint at the G_2/M-phase and suppression of apoptosis results in a small fraction of proliferating cells with a tendency for polyploidisation.

Material and Methods

Tissue specimens

Tumour specimens from 42 different patients were retrieved from the files of the Leiden University Medical Centre (LUMC). A subset of these cases was reported in an earlier study.[10] Tumour samples were fixed in 4% buffered formalin, embedded in paraffin and stained with hematoxylin-eosin for routine pathological diagnosis. Remaining tumour tissue was snap-frozen in isopentane and stored at -80 °C.

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Clinical data recorded for all cases included familiarity and SDHD mutational status,[12] location, multicentricity and age of onset. Tumour volume was calculated from the macroscopic dimensions as described in the pathological reports with the following formula: $4/3\pi$ (length/2 x width/2 x height/2) approaching the volume of an oval shaped object.

Flow cytometry

Measurement of cellular DNA content from paraffin embedded tissue or suspensions from frozen material were performed as described with some modifications.[10] The percentage of tumour cells was estimated by microscopic examination of haematoxylin and eosin-stained 4 μm serial sections. Samples were stained with propidium iodide and measured on a FACSCalibur flow cytometer (Becton Dickinson, Mountain View, CA, USA) using Modfit L.T. V2.0 (Verity Software House Inc., Topsham, ME) for data acquisition. Typically, a minimum of 10.000 events was measured from each sample. Tumours were classified as aneuploid when DNA indices were in the hyperdiploid-hypotetraploid range (>1.05 or < 1.95). Tumours with a DNA index between 1.95 and 2.05 were classified as tetraploid. Some tumours with a diploid G0,1 fraction contained an elevated G_2/M fraction not accompanied by a concomitant increased S-phase fraction. These tumours were classified as "diploid with elevated G_2M fraction" when the G_2M fraction was > 10% which exceeds the G_2M fraction in paediatric tonsils that have significant proliferative activity (data not shown).

Immunohistochemistry

Immunohistochemistry was performed as described previously[13] with a modification involving the use of the Envision® polymer technique (DAKO, Glostrup, Denmark) according to the manufacturers instructions. This was done to avoid endogenous mitochondrial biotin staining,[14] which is a problem with the standard biotin-streptavidin technique. The primary monoclonal antibodies are listed in Table 4.1. The expression of Ki-67 was quantified using computerised field image analysis as described previously with modifications.[15] Briefly, both the Ki-67-positive area (total number of pixels with optical densities exceeding the background) and the total (nuclear) area stained by hematoxylin were measured in 25 high power (400x) microscopic fields. The Ki-67 positive nuclear fraction is expressed by the ratio: total Ki-67-positive area/ total hematoxylin- positive area. The

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Table 4.1: primary antibodies applied

antibody	manufacturer	dilution
Ki-67 (Mib-1)	Boehringer Mannheim, Germany	1:100
P53 (Do-7)	Dako, Glostrup, Denmark	1:250
P21^{waf}	Calbiochem, Cambridge, UK	1:125
Bcl-x$_l$	Transduction, Lexington, KY, USA	1:200

Antibodies diluted in 1% phosphate buffered saline with 1% bovine serum albumin (PBS/BSA).

immunohistochemical results obtained with the other antibodies were evaluated qualitatively.

Two malignant cases with associated metastatic lymph nodes, which were included in the immunohistochemical assays were not examined with computerised field image analysis due to lack of homogeneous tumour tissue and subsequent interference with connective tissue or lymphatic components (metastasis).

TUNEL assay

Fragmented DNA was visualised with the In Situ Cell Death detection Kit (Roche Diagnostics, Mannheim, Germany), as described previously, with modifications.[16] Briefly, deparaffinated sections were pretreated in 0.1 M citrate buffer with microwave irradiation (60 s, 750W) and with blocking buffer (0.1 M Tris-HCl, 3% BSA and 20% NCS). Next, the specimens were incubated with labeling mixture-containing fluorescein at 37ºC and after 60 minutes the reaction was terminated by transferring the sections to stopbuffer (300 mM sodium chloride, 30 mM sodium citrate) for 15 minutes. Incubation with blocking buffer was repeated and Fluorescein was labeled by Peroxidase-conjugated Rabbit anti-fluorescein isothiocyanate (DAKO, Glostrup, Denmark), diluted with blocking buffer, for 30 minutes. The sections were stained with diaminobenzidine chromogen for 1 minute and counterstained with hematoxylin. Appropriate positive and negative controls were included in the assay.

Statistical analysis

Differences in Ki-67 proliferation-index and mutation-positive and -negative tumours were tested between subsets of tumours (mutation-status, ploidy and tumour-volume) using SPSS for Windows 9.0 (SSPS, Chicago, IL).

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Results

Clinicopathological findings

The clinical and pathological characteristics are summarised in Table 4.2 (see later in this chapter). Forty cases were clinically benign. Two tumours (8 and 27) were classified as malignant with documented regional lymph node metastasis in the neck. Twenty-five cases had a familial background, and 12 tumours were classified as sporadic tumours. In 5 cases the family history was uncertain. Patients with positive family history often displayed multicentricity whereas this occurred in only two sporadic cases.

SDHD mutation analysis revealed two Dutch founder mutations in most cases with a positive family history: the Asp92Tyr mutation in exon 3 and the Leu139Pro mutation in exon 4.[12] Two tumours were linked to the PGL2 locus on chromosome 11q13 and no SDHD mutations were detected in these cases.[6] Of all sporadic tumours that were screened for mutations, two cases harboured SDHD mutations (tumours 27 and 29), 1 of which (tumour 27) was a founder mutation. Interestingly, neither patient displayed multicentricity but the tumour harbouring the founder mutation (tumour 27) proved to be malignant. The other malignant case also belonged to a PGL1 family. No mutations were found in the five cases with an uncertain family history, although four patients exhibited multicentricity, a characteristic suggesting a hereditary background.[12]

Patient age at treatment ranged from 20 to 70 years (median 40.5 years). Tumour volume varied between 0.4 and 127,7 cm^3 (mean 14,9 cm^3).

DNA ploidy

Aneuploid cell populations were detected in 52% (22/42) of the tumours. The majority of the tumours (14; 33,3%) had DNA indices in the hyperdiploid-hypotetraploid range whereas 8 tumours (19%) were tetraploid (Figure 4.1). Remarkably, 13 diploid tumours (30.9%) showed, an elevated G_2/M fraction in addition to a diploid G0,1 peak. Only a minority of cases (7; 16,7%) showed a normal, diploid DNA content distribution. Because of the generally very low S-phase (<5%) this fraction could not be accurately calculated due to interference with background signals. Representative DNA histograms are shown in Figure 4.2.
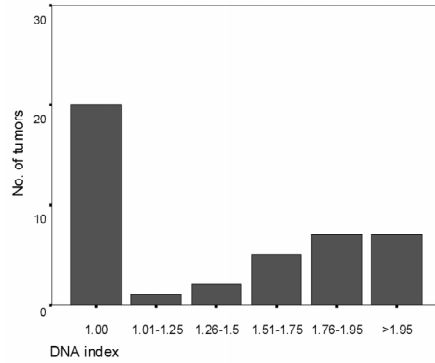
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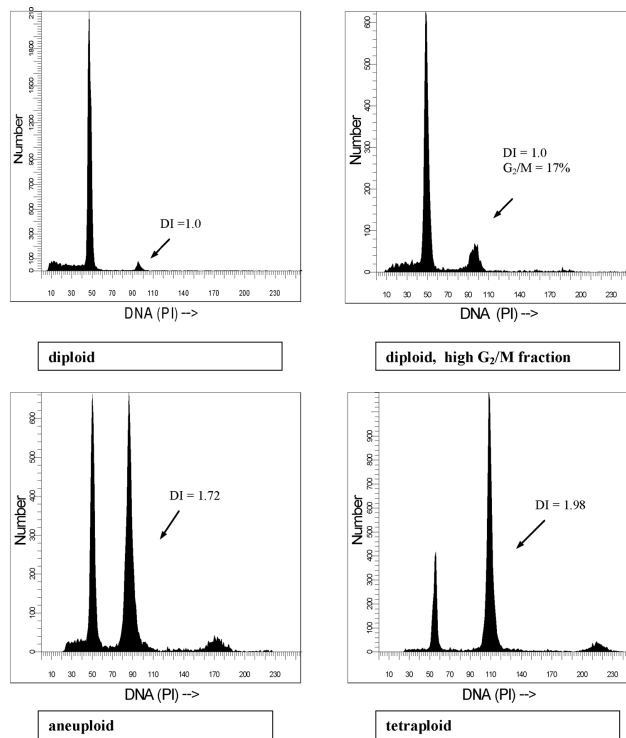**Figure 4.1**: Distribution of DNA indices as determined with flow cytometry

Figure 4.2: Representative DNA histograms of carotid paragangliomas. Horizontal axis: relative DNA values, Vertical axis: number of nuclei counted. DI = DNA index, $G_2/M\%$ = percentage of nuclei in G_2/M phase

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Histopathological findings

All paragangliomas displayed a dominant population of medium to large chief cells frequently organised in clusters designated as Zellballen interspersed with stromal tissue (Figure 4.3A). Occasionally the chief cells formed cords of tumour cells or lacked a clear organisation. Nuclei of chief cells frequently exhibited prominent nucleoli and pleomorphism. Multinucleated cells or giant nuclei were often present (Figure 4.3B). Mitotic figures were rare and occasionally detected with Ki-67 immunohistochemistry (Figure 4.3C). Histological signs of apoptosis such as apoptotic bodies were never seen. The presence and configuration of sustentacular cells varied from a typical localisation in Zellballen to virtually absent in some tumours.

Immunohistochemistry

Ki-67: In all tumours, only small numbers of chief cells showed positive nuclear staining of the Ki-67 antigen (Figure. 4.3D). The two malignant tumours and their metastases showed similar staining patterns as the benign cases. The fraction of Ki-67-positive nuclear area varied from 0.02% to 1.74% (average 0.52% ± 0. 4%). In the majority of tumours (n =35) the Ki-67-positive nuclear area fraction was < 1% (Table 4.2). For comparison, a larynx carcinoma and a breast carcinoma yielded values of 29.27± 8.27% and 24.0% ± 7.71%, respectively.

Tp53 :Tumours showed either negative or faint nuclear p53 protein staining in chief cells (Figure 4.3E). Occasionally, a single cell showed strong nuclear staining. Positive control tissue invariably showed strong nuclear staining (data not shown). Only one case showed a higher fraction of p53-positive nuclei of chief cells.

P21^{waf} :Nuclear staining was observed in moderate numbers of chief cells in 40 of the 42 tumours (Figure 4.3F). In addition, 13 cases showed clustering of positive chief cells (Figure 4.3G). There was no apparent topological relation between these clusters and Ki-67 or p53 expression in adjacent histological sections.

Bcl-x$_l$: Bcl-x$_l$ staining was positive in 40 of 41 cases examined. A strong granular cytoplasmic staining was seen in 22 of 41 tumours whereas more diffuse cytoplasmic staining was seen in 18 tumours (Figure 4.3H). Sections of a neuroblastoma used as a positive control showed strong staining of neoplastic cells and adjacent muscle (data not shown).
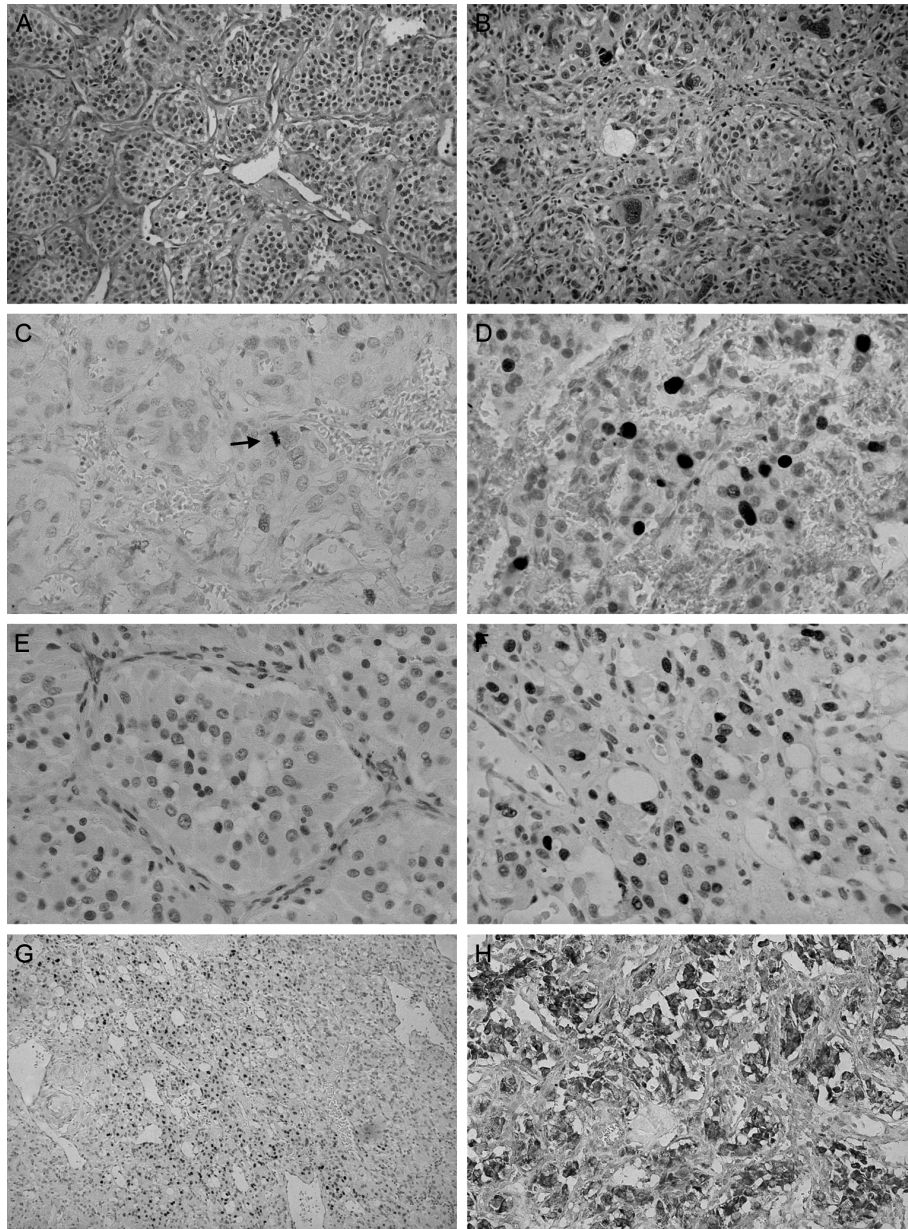
Figure 4.3: Histology and immunohistochemistry of head and neck paragangliomas. A: HE-staining 25x, B: Nuclear pleomorphism and giant nuclei, HE-staining 50x, C: mitotic figure (black arrow), Ki-67 staining 100x, D: scattered nuclear staining of Ki-67, 100x, E: faint nuclear staining of Tp53, 100x, F: nuclear staining of p21^{waf}, 100x, G: clustering of p21^{waf} staining, 12.5x, H: strong granular cytoplasmic staining of Bcl-x$_l$, 50x.(Colour image on page 142).

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TUNEL- assay

Analysis was done in a subset of ten randomly selected tumours. Only occasionally apoptotic bodies were visible whereas the positive control tissue (epidermis) showed apoptotic bodies at the expected locations.

Correlation between variables

Of all variables, only ploidy (aneuploid versus diploid) showed a significant correlation with hereditary status (p = 0.031, cross-tabulation). Aneuploid tumours were more frequent in cases with hereditary background or proven SDHD germline mutations.

Discussion

In this study we have investigated some factors that may account for the remarkable indolent growth of head and neck paragangliomas, with attention to possible differences between hereditary and sporadic tumours. The proliferative activity (growth fraction) as determined by computerised image analysis of immunohistochemical Ki-67 expression was found to be < 1% in most cases (average 0.52%). This is in accordance with the slow growth of these tumours. Two trends were observed. First, the tumour volume is smaller in hereditary tumours at resection, possibly reflecting the general greater awareness of family members and physicians. Secondly, all tumours with a Ki-67 index > than 1% were hereditary cases, but showed an intermediate volume distribution. This trend is in accordance with the positive correlation between size and tumour doubling time suggesting a model of retarded growth.[2]

Comparison with our own Ki-67 measurements from head and neck carcinomas and breast carcinomas and with published data on malignant tumours[17] shows a dramatically low growth fraction in head and neck paragangliomas. Other studies have reported similar low Ki-67 proliferation indices for paragangliomas of approximately 5% based on the methods employed.[18,19]

We and others previously reported that a sizeable proportion of the head and neck paragangliomas is DNA-aneuploid.[10,20] In the present study on an extended series of tumours abnormal DNA-content profiles were found for approximately 80% of the cases with more than 50% of the tumours containing clearly aneuploid or tetraploid DNA-stemlines. Interestingly, aneuploidy was more frequently detected in hereditary tumours (p = 0.031). As in our previous study, we noticed an unusual

tumor	location	family history	mutation	ploidy	DNA index	volume [cm³]	multicentricity	behaviour	Ki-67 [%]
1	cbt	PGL1	D92Y	a	1.83	5.89	cbtl. vntl		0.55
2	cbt	PGL1	D92Y	a	1.75	2.45			0.84
3	cbt	PGL1	D92Y	a	1.41	6.29	vntl. vntr.pheo		0.65
4	cbt	PGL1	D92Y	a	1.69	4.71	cbtr		0.02
5	cbt	PGL1	D92Y	a	1.85	3.46	vntl		0.54
6	cbt	PGL1	L139P	a	1.79	4.91	cbtl		0.16
7	cbt	PGL1	D92Y	a	1.72	2.57			1.01
8	cbt	PGL1	nd	a	1.94	33.5	vntr	m	-
9	cbt	pos	nd	d	1.00	0.38	vntl		0.02
10	cbt	PGL1	D92Y	d	1.00	1.44			0.65
11	cbt	PGL1	D92Y	G2	1.00	8.18	cbtr. gttl		0.22
12	cbt	PGL1	D92Y	G2	1.00	25.14			0.44
13	cbt	PGL2	neg	G2	1.00	6.87	jttl		0.23
14	cbt	PGL1	L139P	G2	1.00	3.14	cbtl. vntl		0.05
15	cbt	PGL1	L139P	G2	1.00	0.65	cbtr. jttr		0.08
16	cbt	PGL1	D92Y	G2	1.00	4.19	vntl		0.48
17	cbt	PGL1	D92Y	t	2.00	15.71	cbtl. vntl		0.11
18	cbt	PGL1	D92Y	t	2.00	28.28	vntl		0.39
19	cbt	PGL1	D92Y	t	1.98	1.6			0.27
20	cbt	PGL1	D92Y	t	2.00	3.77			0.58
21	cbt	PGL1	D92Y	t	2.00	9.82			0.34
22	cbt	PGL1	D92Y	t	2.00	7.26	jttl. vntr		1.74
23	cbt	spor	neg	a	1.84	4.19			1.7
24	cbt	spor	neg	d	1.00	127.66			0.84
25	cbt	spor	nd	G2	1.00	14.14			0.75
26	cbt	spor	neg	G2	1.00	18.86			0.27
27	cbt	spor	D92Y	G2	1.00	10.48		m	-
28	cbt	spor	nd	t	1.95	14.14			0.17
29	cbt	spor	G6825 insC	t	2.02	5.89			0.61
30	cbt	spor	neg	a	1.31	7.76			0.48
31	cbt	?	neg	d	1.00	31.43	cbtr		0.09
32	cbt	?	neg	G2	1.00	13.09	vntr		0.14
33	cbt	?	neg	G2	1.00	5.5			0.51
34	cbt	0	neg	G2	1.00	18.86	cbtr. vntr		0.07
35	cbt	0	neg	a	1.53	50.28	cbtr		0.83
36	jtt	PGL1	L139P	d	1.00	2.58	cbtl		1.31
37	jtt	spor	neg	G2	1.00	0.52			0.81
38	vnt	PGL1	D92Y	a	1.73	14.14	cbtl. jttl		1.45
39	vnt	PGL2	neg	d	1.00	1.26	cbtl. cbtr. vntr		0.39
40	vnt	spor	nd	a	1.87	70.71			0.15
41	vnt	spor	neg	d	1.00	35.35	cbtl		0.62
42	vnt	spor	neg	a	1.18	0.4	cbtr		0.15

Table 4.2: Clinical and pathological characteristics of paragangliomas: cbt: carotid body tumor, jtt: jugulo-tympanic tumor, vnt: vagal nerve tumor, pheo: pheochromocytoma, ()r and ()l: right and left side, PGL1: positive family history (PGL1 locus 11q23), PGL2: positive family history (PGL2-locus 11q13), pos: positive family history, spor: sporadic, ?: uncertain family history, D92Y, L139P and G6825 insC : SDHD mutations, nd: mutational status not determined, neg: no sdhd mutation detected, a: aneuploid, d: diploid, G2: high G2/M fraction, t: tetraploid, m: malignant.

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high number of diploid tumours with an elevated G_2/M fraction not accompanied by a concomitant increased S-phase fraction. In the present series this comprised 31% of all cases. Since the DNA profiles showed no additional population in the 8n region, these elevated G_2/M populations likely represent cells arrested in G_2 phase of the cell cycle. However tetraploidization of a small subpopulation cannot entirely be excluded. The suggested G_2/M arrest accounting for the elevated G_2/M fractions in diploid tumours may signify a still functional mitotic checkpoint. The clearly tetraploid tumours could represent progression towards chromosome instability after inactivation of this checkpoint ultimately resulting in overt aneuploidy.[21,22]

The tumour suppressor gene Tp53 plays a central role in cellular stress response signalling and can induce programmed cell death and cell cycle arrest. The vast majority of head and neck paragangliomas did not show strong nuclear p53 expression indicative for mutations.[23] The protein was generally absent or showed faint nuclear expression. It is unclear whether this staining pattern represents functional p53 activation. Only one case displayed stronger immunoreactivity. The percentage of Ki-67-positive chief cells in this case was near the average (0.6%), and clinical behaviour was benign. These results indicate that most likely p53 is not mutated in the vast majority of head and neck paragangliomas.[24]

Associated with p53 activity is the expression of the cell cycle inhibitor p21[waf]. This protein is capable to arrest cell cycle progression at the transition from G1 to S phase (G1-S) and G_2 to M phase (G_2-M) through binding and subsequent inhibition of cyclin-CDK complexes.[22] Activity of p21[waf] is primarily regulated through p53 and this pathway is generally believed to be an important mechanism through which p53 exerts cell cycle control.[23] We found scattered p21[waf] expression in most paragangliomas. An interesting observation was the occasional clustering of p21[waf]-positive cells. The expression of p21[waf] in tumours could indicate a cellular stress response in chief cells mediated through functional p53. Mitochondrial dysfunction due to inactivating mutations in complex II proteins could be the cause of (oxidative) cellular stress. Interestingly, it has been shown that induction of p21[waf] can result in a G_2/M arrest, but after prolonged induction, arrested cells can reinitiate in the cell cycle and have a tendency towards polyploidisation.[22,25]

In addition to cell cycle arrest, the p53-stress response may result in activation of apoptosis.[26] However morphological stages of apoptosis were not observed and no DNA-strand breaks were detected with the TUNEL assay. This implies that no significant apoptotic activity is present in paragangliomas. The strong cytoplasmic expression of the anti-apoptotic Bcl-x$_L$ protein in chief cells may explain this absence.

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This protein is located at the outer mitochondrial membrane and can inhibit apoptosis through blocking the release of mitochondrial pro-apoptotic proteins.[27,28] Recently it was shown that Bcl-x can inhibit apoptosis during oxygen deprivation through stabilisation of mitochondrial membranes.[29] SDHD mutations may influence apoptotic pathways through impairment of mitochondrial function or (indirectly) lead to an increase of Bcl-x_l protein.[8,29] This mitochondrial association may explain the granular staining pattern of Bcl-x_L in >50% of the tumours. Less-optimal fixation could account for the diffuse staining in the other cases. Interestingly, results from in vitro experiments show that Bcl-x_L overexpression in murine Fl cells after induced mitotic spindle damage leads to a prolonged p53-dependent G_2 arrest with impairment of apoptosis and eventually progressive polyploidisation of the cells.[30] Investigators have hypothesised that Bcl-x_L allows the cells to survive to a point after which p53 is downregulated perhaps by adaptation of the checkpoint pathway. Cells then would continue to proceed through the cell cycle leading to polyploidisation.

Based on our findings and the foregoing considerations, we propose a model for tumourigenesis in paragangliomas (Figure 4.4). We assume that mitochondrial dysfunction generates oxidative stress and activates an unknown downstream mitogenic signalling pathway leading to aberrant cell cycling. Replication stress may activate cell cycle checkpoints causing a G_2/M arrest.[21,22] But the apoptotic pathway for eliminating these arrested cells is impaired or even blocked due to high expression of Bcl-x_L.[27] This could explain the apparent absence of a selection pressure for p53 inactivation, because one of the major functions of this protein is to initiate apoptosis in cells that have acquired critical DNA damage.[23,26] Due to their enhanced survival, the arrested cells accumulate. But when the checkpoint decays, some cells terminate their arrest and reinitiate replication and division. In the presence of functional p53 and Bcl-x_L this process eventually results in progressive polyploidisation as has been demonstrated with other cell lines in vitro.[22,25,30] Although
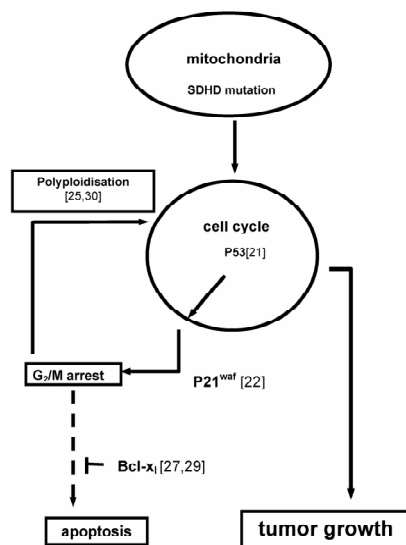

Figure 4.4: Schematic model for tumourigenesis in paragangliomas. References in square parentheses.

the aneuploid tumours have marginally elevated proliferation rates, the growth of these tumours remains low. Therefore the likelihood for acquiring additional mutations that could accelerate tumour progression is probably small. The low proliferation rate may even be partly the result of high levels of Bcl-x$_l$, because anti-apoptotic Bcl proteins can delay cell cycle entry at the transition from the G$_0$ phase into the S phase.[31] Thus this combination of events may lead to a slowly proliferating neoplastic population of cells with progressive polyploidisation and a low malignant potential.[32]

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Chapter 5

Multiparameter DNA flow-sorting demonstrates diploidy and SDHD wild-type gene retention in the sustentacular cell compartment of head and neck paragangliomas: chief cells are the only neoplastic component

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Abstract

Head and neck paragangliomas are considered to be biphasic tumours, composed of two distinct cell types: chief cells and sustentacular cells. A substantial number of these tumours show mutations in the SDHD-gene located at chromosome 11q23. Although there is general agreement that paragangliomas are a neoplastic proliferation of chief cells, the nature of sustentacular cells is still a matter of debate. To clarify the nature of sustentacular cells further, multiparameter DNA flow cytometry was performed utilising S-100 labelling as a selective marker of the sustentacular fraction simultaneously with DNA content measurement in 6 head and neck paragangliomas. S-100 positive fractions and other tumour-cell populations were flow-sorted and restriction-digestion analysis for presence of SDHD mutations was performed on each fraction. Flow cytometry demonstrated that the S-100 labelled cells were diploid. Restriction-digestion analysis in informative cases revealed retention of wild type SDHD-allele in S-100 positive fractions and loss of wild-type allele in S-100 negative fractions. These data strongly suggest that sustentacular cells should be regarded as a non-neoplastic cell population that may be induced as a tumour specific stromal component by chief cells.

Introduction

Head and neck paragangliomas are rare hypervascular tumours that originate from the neural crest derived paraganglia, which are associated with the autonomic nerve system. An important feature is the strong hereditary background in many patients with autosomal dominant transmission and genomic imprinting through the maternal line.[1] Recent studies have identified genes encoding for subunits of complex II of the mitochondrial respiratory chain as susceptibility genes for paragangliomas and pheochromocytomas. These genes, SDHB, SDHC and SDHD were found to behave like bona fide tumorsuppressor genes.[2] Germline mutations in the SDHD-gene on chromosome 11q23, encoding for an anchor-subunit of complex II are responsible for the majority of hereditary cases in the Dutch population.[3]

Histologically, the normal paraganglion is organized into typical cell nests (Zellballen), which are composed of clusters of chief cells that are surrounded by supportive sustentacular cells at the periphery. In the normal carotid body, both cell types are thought to derive through differentiation from common progenitor

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neural crest cells that migrate along the glossopharyngeal nerve.[4] Ultrastructurally, the sustentacular cells envelope the nerve endings and protrude between chief cells with delicate cytoplasmic extensions.[5] The precise function of sustentacular cells is unclear although they possibly assist the chief cells in the process of chemosensing and may serve as a supportive element for the nerve endings.[6,7] The tumours frequently resemble normal paraganglia with the characteristic organization of chief cells and sustentacular cells into Zellballen. Although there is consensus that paragangliomas constitute neoplastic proliferations of chief cells, there has been considerable debate concerning the nature and role of sustentacular cells.[8-10] Variable populations of sustentacular cells, which are readily detected with S-100 immunohistochemistry, can be present and several studies describe an inverse correlation between the number of sustentacular cells and the clinical behavior of the tumour.[8,11-15] Two concepts may explain the dual composition of paragangliomas and the nature of sustentacular cells. First, the sustentacular cells may constitute an additional population of neoplastic cells derived from the same lineage as the chief cells, and paragangliomas could therefore be considered to be biphasic tumours.[5,11,12] On the other hand, sustentacular cells could represent a reactive stromal component, which is induced by the neoplastic chief cells.[16]

In order to clarify the dual composition of paragangliomas, the nature of sustentacular cells was further characterized in a series of 6 head and neck paragangliomas. We developed a flow cytometric procedure for the identification and isolation of sustentacular cells using S-100 labeling. The ploidy of the S-100 fraction of a tumour with abnormal DNA-content could thus be evaluated. After flow-sorting the S-100 positive fractions, we investigated the sustentacular cell DNA for loss of heterozygosity (LOH) of the wild type SDHD allele. The results provide support for the concept that sustentacular cells are of stromal origin and consequently chief cells should be considered to be the exclusive neoplastic component of paragangliomas.

Material and Methods

Tumor samples
Paraffin wax-embedded tissue samples of head and neck paragangliomas were retrieved from the archives of the Department of Pathology. The tissue specimens used in this study were archived and retrieved according to the guidelines of the Dutch Federation of Research Associations (FEDERA). The collection of clinical and

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family data from paraganglioma patients has been approved by the Institutional Review Board. Relevant clinicopathological data were collected including tumour location, family history and prior ploidy status. The histological appearances of all cases and controls were reviewed. Tumours were selected that (i) contained large populations of sustentacular cells, which were detected with routine S-100 immunohistochemistry and (ii) harbored tumour cell populations with an aberrant DNA-content as determined with flow cytometry (see later in this section). In order to validate the experimental procedures, a cutaneous transit metastasis of malignant melanoma and an appendiceal carcinoid tumor were selected as positive controls for S-100 expression.[17,18] In the paragangliomas, the presence of significant populations of other S-100 positive cells such as dendritic cells or normal neural tissue was excluded histologically prior to flow cytometry experiments.

Immunohistochemistry
A standard three-step indirect immunoperoxidase technique was performed on 4 μm thick paraffin wax sections, mounted on APES pre-coated glass slides with modifications.[19] Briefly, after dewaxing and rehydration, the sections were subjected to a trypsin-based antigen retrieval method for 20 min at 37°C [0.1% trypsin, 10 mM Tris (pH 7.4), 0.1% $CaCl_2$]. Next sections were incubated for 1 hour with an anti-S-100 monoclonal antibody (Chemicon, Harrow, UK) or appropriate negative controls, followed by incubation steps with biotinylated rabbit anti-mouse immunoglobulins and biotinylated peroxidase-streptavidin complex (ABC-kit, DAKOCytomation, Glostrup, Denmark). Assessment of S-100 expression was evaluated semi-quantatively using a scoring regimen as described previously.[20] Briefly, sections were scored for staining intensity (**0**, no staining; **1**, weak; **2**, moderate; **3**, strong) and the percentage of tumour containing stained sustentacular cells (**0**: 0%, **1**: 1%-24%, **2**: 25%-49%, **3**: 50%-74%, **4**: 75%-100%). Both scores were added to generate a total score.

Cell isolation and staining
Initially, cellular DNA content was measured from paraffin wax-embedded tissue as described previously.[21] Selected samples were processed for multiparameter DNA flow cytometry using an improved Hedley method.[22]
Cell isolation: Multiple 50 μm thick sections from paraffin wax-embedded tumour blocks were cut and collected in small baskets prepared from polymer gauze with 85 μm pores, allowing easy manipulation in reaction tubes. Sections were

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deparaffinized and preincubated in 0.1 M citrate buffer (pH 6.0) for 5 minutes, followed by incubation in citrate buffer at 80 °C for 80 min. After cooling and washing, the sections were subjected to antigen retrieval through enzymatic digestion with trypsin at 37 °C for 20 minutes. Next the dissociated cells were separated from the remnants of the sections by vortexing thereby forcing the cells through the pores of the baskets in the surrounding suspension. After removal of the baskets, the suspended cells were washed through centrifugation at 400g for 5 min and resuspension of pelleted cells in fresh phosphate buffered saline with 1% bovine serum albumin.

Staining:The concentration of suspended cells was determined in a haemocytometer using tryptan blue as counter stain and the suspension was aliquoted in different reaction-tubes, each containing 1.10^6 cells. Cells were centrifuged and incubated with 100 μl S-100 antibody or irrelevant lgG2A immunoglobulins and incubated overnight at 4 °C. Primary antibody (anti-S-100) concentration was optimised for flow cytometry after extensive pilotstudy's. Next, cells were washed and incubated with a 100 μl solution of FITC labelled goat anti mouse Ig immunoglobulins (DAKOCytomation, Glostrup, Denmark) for 1 hour at 4 °C.

Labelling: Cells were washed twice and incubated with a DNA staining solution containing 10mM DRAQ5 and 0.1% RNAse (Sigma).[23,24] After overnight storage at 4 °C, samples were analysed by flow cytometry.

Flow Cytometry and – Sorting

For each measurement, data from 10,000 single cell events were collected using a Standard FACScalibur (BD Biosciences, San José, CA) flow cytometer. FITC (FL1, BP 530/30 nm) fluorescence was collected in the logarithmic mode. DRAQ5 fluorescence was collected in the linear mode using a 670 nm LP-fitter (FL3). Only the blue 488 nm laser line from the Argon-ion laser was used for excitation. A live gate was set using the FL3-A vs. FL3-W doublet discrimination module (DDM) allowing single cell measurements. Data were analysed using the WinList 5.0 and ModFit 3.0 software packages (Verity Software House, Inc, Topsham, ME, USA). S-100 positive as well as S-100 negative cell populations were flow-sorted using a FACSVantage flow-sorter (BD Biosciences, San José, CA, USA) applying the FACSCalibur filter settings for FITC and DRAQ5 fluorescence, respectively.

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DNA isolation, mutation detection and LOH analysis

The sorted cell populations and non-sorted control samples were subjected to Protease-K digestion to purify nuclear DNA as described before.[25] Approximately 250 nuclei were digested in 1μl buffer. The resulting solutions containing purified DNA were heated for 10 min at 100 °C to inactivate proteases and DNAse and served as templates for polymerase chain reactions (PCRs). Tumour DNA was screened for the most common Dutch SDHD founder mutation (Asp92Tyr) by restriction digestion as described previously.[3] Single-exon PCR analysis and mutation detection by restriction endonuclease digestion was carried out using mutation-flanking primers (F3; CTTTTATGAATCTGGTCCTTTTTG, R3; CAACTATATTTGGAATTGCTATAC) for exon 3 of the SDHD-gene.

Amplified exon-3 products were digested with the restriction enzyme *RsaI* to detect the restriction site created by the Asp92Tyr mutation according to the manufacturer's recommendations. Samples were analysed on a 2% agarose gel. The presence of SDHD mutations and allelic imbalance (retention or loss) of the wild type gene in non-sorted and sorted fractions was determined through examination of digestion patterns. DNA extracted from peripheral blood samples from a patient with a confirmed Asp92Tyr-mutation and a non SDHD-linked patient served as control-samples to validate the PCR-process and restriction digestion.

RESULTS

Immunohistochemistry

Six cases of head and neck paragangliomas were selected on the basis of ploidy and immunohistochemical expression patterns for S-100. The clinicopathological characteristics are listed in Table 5.1. All cases behaved in a clinically benign

tumor	location	fam history	S-100	ploidy	S-100 fraction	digestion exon 3	allelic (im)balance
1	cbt	positive	4	a	d (2n)	*RsaI* gain	S-100: retention wild type aneuploid: loss wild type
2	vnt	sporadic	7	a	d (2n)	no mutation	
3	cbt	positive	6	a	d (2n)	no mutation	
4	jtt	unknown	6	G2/M	d (2n)	*RsaI* gain	S-100: retention wild type G2/M: loss wild type
5	cbt	positive	7	a	d (2n)	*RsaI* gain	S-100: retention wild type aneuploid: loss wild type
6	cbt	unknown	7	G2/M	d (2n)	no mutation	

Table 5.1: *cbt*: carotid body tumour, *vnt*: vagal nerve tumour, *jtt*: jugulotympanic tumour, *S-100*: scores of immunohistochemical staining of sustentacular cells as described in methods section, *a*: aneuploid, G_2/M: high G_2/M fraction, *d(2n)*: diploid with 2n DNA content.

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fashion. Each tumour contained positive populations of sustentacular cells and negligible numbers of other positive cells (figure 5.1 A). The expression patterns were specific and no background staining could be detected. Tumour cells were also strongly positive for S-100 in the appendiceal carcinoid tumour and cutaneous transit metastasis of malignant melanoma (figure 5.1B). In the melanoma sections however, some S-100 positive neural tissue was also present.
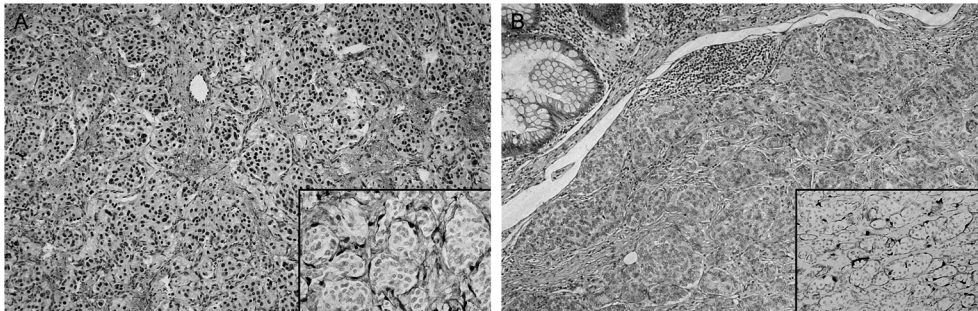

Figure 5.1: *A)* Light-micrograph displaying characteristic Zellballen pattern in paraganglioma, HE 25x, *insert*: immunohistochemical staining showing positive sustentacular cells in paraganglioma, S-100; 100x *B)* Light-micrograph of appendicular carcinoid, HE; 25x, *insert*: immunohistochemical staining showing positive spindle cells in carcinoid, S-100; 25x. (Colour image on page 143)

Flow cytometry and - sorting

Multiparameter flow cytometric analysis of the cutaneous transit metastasis of melanoma showed an aneuploid population of tumour cells with increased fluorescence after S-100 labelling as well as fluorescence of a diploid population most likely representing neural elements (figure 5.2A). Four paragangliomas showed aneuploid populations and 2 tumours contained elevated G2/M fractions. Two-parameter flow cytometry, revealed S-100 positive diploid subpopulations in all paragangliomas and in the carcinoid tumour. No S-100-positivity was detected in the aneuploid fractions or the increased G2/M fractions of the two diploid paragangliomas, except for some scattered signals representing cell-aggregates (figures 5.2B-D). Subsequently, the S-100 positive diploid populations and populations with aneuploid DNA content or elevated G2/M fractions were sorted from samples of all 6 paragangliomas (figure 5.2D).
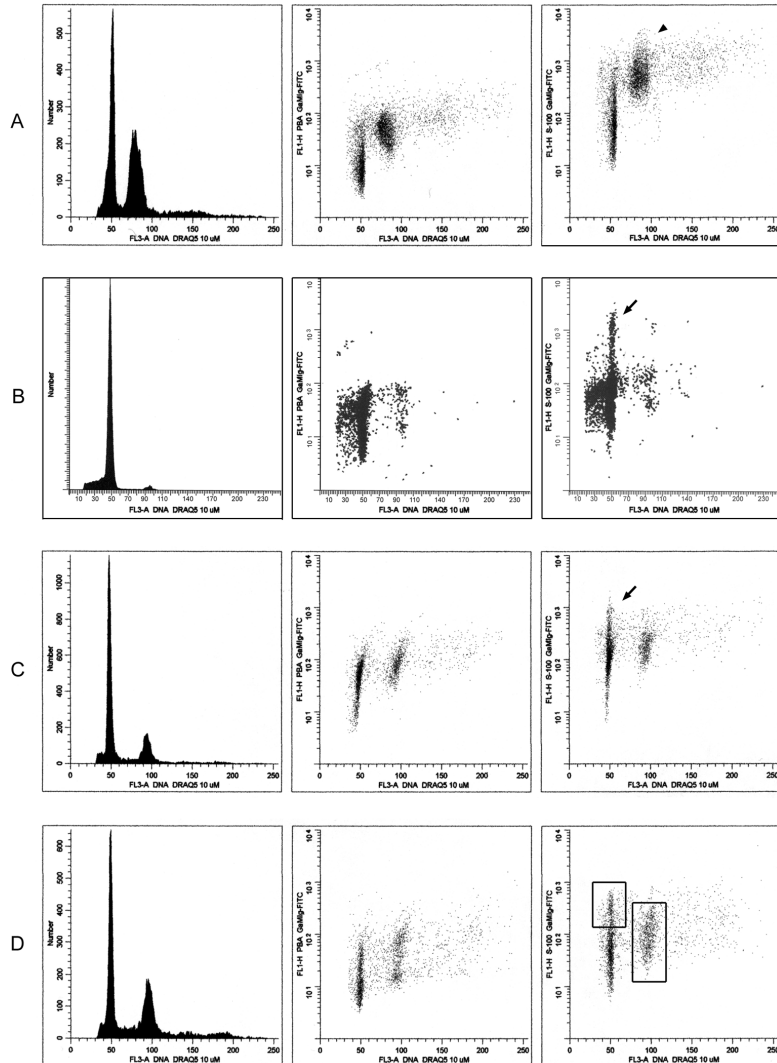
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Figure 5.2: Flow cytometric results. Row *A*) cutaneous transit metastasis of malignant melanoma, *B*) appendicular carcinoid, *C & D*) head and neck paragangliomas 3 (C) and 5 (D). *Left column*: 1-parameter DNA content histograms: aneuploidy of melanoma and both paragangliomas, diploid carcinoid. *Middle column*: isotype control (Ig2$_a$ immunoglobuline) vs DNA content. *Right column*: 2-parameter dot plots showing S-100 expression (vertical axis) vs DNA content (horizontal axis). Comparison with the isotype control shows that the aneuploid melanoma cells are strongly positive for S-100 by the increased fluorescence in the aneuploid population (arrowhead). Simultaneous increase in the diploid fraction is due to neuronal tissue present in the epidermis. The carcinoid and paragangliomas show S-100 positivity of the diploid cell populations (arrows) whereas the aneuploid tumour populations are negative. Squares and rectangles in histogram of paraganglioma #5 (D) indicate the gates set for sorting of the different cell populations.

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Restriction analysis

Three cases were informative after digestion of exon 3 of the SDHD-gene with restriction enzyme *RsaI* and showed a heterozygous Asp92Tyr mutation in the unsorted sample. In each case the sorted S-100- positive fraction had retained the wild type allele whereas the sorted aneuploid or G2/M-fractions showed an allelic imbalance indicating loss of the wild type allele (figure 5.3).
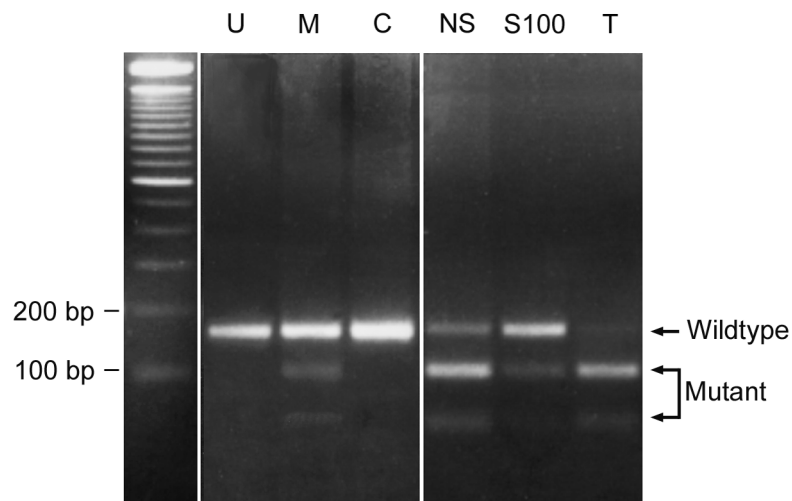

Figure 5.3: Retention and loss of wild type *SDHD* allele in sorted cell-populations from head and neck paraganglioma # 4. After PCR amplification of exon 3, digestion was performed with restriction enzyme *RsaI* to detect the Asp92Tyr mutation. The mutation creates a restriction site within the 200 bp fragment resulting in two fragments of 132 and 68 bp.
The digest from the non-sorted population shows a wildtype and mutant allele. The diploid S-100 fraction shows retention of the wildtype allele whereas in the sorted tumour population loss of the wild type allele is observed. Control-digestions are obtained from peripheral blood samples *U*: undigested fragment, *M*: control digestion of fragment with Asp92Tyr mutation, *C*: control digestion of fragment without Asp92Tyr mutation, *NS*: non-sorted population, *S-100*: S-100 positive population, *T*: tumour population.

DISCUSSION

Head and neck paragangliomas are typified by organisation of chief cells and sustentacular cells into Zellballen resembling the architecture of the original paraganglion. Although there is consensus that these tumours represent true neoplastic proliferations of chief cells, the role and nature of the sustentacular cells has been the subject of considerable debate.[5,8,9,12] Some authors consider the sustentacular cells to be a second neoplastic cell type and thus have classified paragangliomas as truly biphasic tumours.[5,11,12] Others regard these cells as a

non-neoplastic stromal component that is induced by the proliferating neuroendocrine chief cells.[16] The recent identification of the SDHD-gene as the major susceptibility gene in hereditary paragangliomas and the demonstration of LOH of SDHD in tumours provide a genetic marker for clarifying the nature of sustentacular cells.[2] In this study we used multiparameter flow cytometry to characterise and isolate the S-100 labelled fractions in paragangliomas with abnormal DNA-ploidy. Because the S-100 protein is a soluble antigen, formalin-fixed and paraffin wax-embedded tissue was utilised in the procedures.[26] Immunohistochemistry was performed to verify that the S-100 positive populations consisted predominantly of sustentacular cells in the selected tumours and therefore these results are not confounded by large populations of other S-100 expressing cells (e.g. dendritic cells). In all cases the S-100 labelled fraction proved to be diploid. After subsequent sorting of various fractions and restriction-digestion analysis, retention of the wild type SDHD-allele in S-100 positive fractions could be demonstrated whereas the primary tumour cell populations displayed allelic imbalance with loss of the wild type SDHD-allele. Thus, these results demonstrate that the sustentacular cells likely represent a reactive non-neoplastic population and their presence or growth may be a response to growth factors secreted by neoplastic chief cells.[27,28] These findings argue against paragangliomas being biphasic and support the hypothesis that these neoplasm's represent a monoclonal expansion of chief cells due to inactivation of the SDHD-gene, which behaves as a classical tumour suppressor gene.[2,21] A similar genetic approach has been followed to track the origin of Schwann cells in neuroblastomas. Using interphase FISH, Ambros *et al* demonstrated that Schwann cells in triploid neuroblastomas were diploid and concluded that they constitute a reactive population of normal cells that invade the neuroblastoma.[29] Katsetos *et al* arrived at a similar conclusion for Schwann cells in peripheral neuroblastomas although this was based on topographical relationships and differences in immuno-histochemical expression rather than on genetic (clonal) markers.[30] These combined data support the hypothesis that secondary cell populations in apparently biphasic tumours of neuroendocrine lineage may generally constitute a reactive stromal component rather than a phenotypically different neoplastic subclone. Although the prevailing opinion is that during embryological development, both the sustentacular cells and chief cells originate from a common neural crest precursor,[4] our data and those from the studies cited above indicate that neoplastic chief cells at least can induce sustentacular cells in the stroma.

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The reported inverse relationship between the number of sustentacular cells present and malignant behaviour of the tumour may reflect a decreasing level of differentiation of chief cells. As the chief cells acquire malignant characteristics, a simultaneous loss off differentiation may occur, resulting in a progressive inability to induce and maintain sustentacular formation. Several studies describe that sustentacular cells are more prevalent in better-differentiated areas of tumours that have maintained their architectural integrity whereas histological loss of differentiation in malignant tumours coincides with reduced numbers sustentacular cells.[5,8,16] Similar relations between tumour differentiation and stromal induction have been observed in maturing peripheral neuroblastomas and differentiating PC 12 cells, which were capable of inducing Schwann cell division in *in vitro* experiments.[27,30]

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Chapter 6

Increased prevalence of catecholamine excess and pheochromocytomas in a well-defined Dutch population with SDHD-linked head and neck paragangliomas

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Abstract

Objective: The aim of this study was to identify the prevalence of catecholamine excess and pheochromocytomas in a well defined population of people with hereditary head and neck paragangliomas.

Methods: A prospective, follow-up protocol was used, including all consecutive patients with documented head and neck paragangliomas with either a positive family history for paragangliomas or a proven SDHD gene mutation. Initial analysis included medical history, physical examination and the measurement of excretion of catecholamines in two 24-h urine collections. In the case of documented catecholamine excess [123]iodinated metaiodobenzylguanidine scintigraphy (^{123}I-MIBG) and magnetic resonance imaging (MRI) were done.

Results: Between 1988 and 2003, 40 consecutive patients (20 male and 20 female) with documented head and neck paragangliomas were screened. Biochemical screening revealed urinary catecholamine excess in 15 patients (37.5%). In nine of these 15 patients a lesion was found by ^{123}I-MIBG scintigraphy. Exact localization by MRI revealed pheochromocytomas in seven of the 15 patients. One of the nine patients had an extra-adrenal paraganglioma. Histopathological examination in a subset of tumours displayed loss of heterozygosity of the wild type SDHD-allele in all cases.

Conclusions: The prevalence of catecholamine excess (37.5 %) and pheochromo-cytomas (20 %) is high in patients with familial head and neck paragangliomas. Therefore, patients with head and neck paragangliomas require lifelong, follow up by biochemical testing for catecholamine excess.

Introduction

Paragangliomas are rare hypervascular tumours originating from neural crest derived paraganglia that are associated with the autonomic nervous system. These paragangliomas can develop anywhere from the neck to the pelvis in locations parallel to the ganglion chain. Normal paraganglia can excrete catecholamines in response to stress or act as chemical sensors around blood vessels[1].

Paragangliomas traditionally have been divided into two separate categories with respect to anatomical location: tumours that are located in the head and neck region and tumours situated in thorax and abdomen.[1] In cases where the adrenal medulla is affected, they are referred to as pheochromocytomas. These two

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categories of paragangliomas also demonstrate different clinical courses. Paragangliomas located in the head and neck most often lack excessive secretion of catecholamines and are mainly detected by effects of tumor growth. They are non-chromaffin tumours derived from the parasympathetic system.[1-3] On the other hand, pheochromocytomas may cause paroxysmal symptoms and hypertension due to the excessive secretion of catecholamines.[4]

In general, head and neck paragangliomas within a hereditary context were reported in about 10-25% of the cases.[5,6] Furthermore, in patients with a positive family history the tumours were more often found to be multicentric and, in 30% of cases, bilateral as opposed to 5% in sporadic cases.[6,7]

Recently, the major susceptibility-genes in hereditary paragangliomas have been identified. Germline mutations were discovered in the genes coding for subunits of mitochondrial complex II succinate dehydrogenase (SDH); SDHD (hereditary paraganglioma type 1(PGL1)) at chromosome 11q23, SDHC (PGL3) at chromosome 1q21, and SDHB (PGL4) at chromosome 1p36.[8] In several studies loss of heterozygosity of wild type SDHD- and SDHB-alleles in paragangliomas and pheochromocytomas could be demonstrated, implicating these SDH-genes as tumoursuppressor genes.[9-11] Another PGL locus, mapped to chromosome 11q13 (PGL2) in a single Dutch family, remains to be characterized further.[12]

A large multibranch Dutch family with hereditary head and neck paragangliomas in the central western region of the Netherlands was identified previously. Using this pedigree a single ancestral mutation was deemed to be responsible for most hereditary paragangliomas in this region in the Netherlands.[13,14] While seeing all these patients for a protocolized endocrinological screening, we suspected an increased prevalence of catecholamine excess or pheochromocytomas. In addition, several studies have presented data on the prevalence of SDH gene mutations among familial and sporadic head and neck paragangliomas and pheochromocytomas, showing significantly more mutations among familial cases.[15,16] Previous reports in the literature demonstrated an increased prevalence of pheochromocytomas among patients with SDHD-linked head and neck paragangliomas of only approximately 5 %.[13] However, this prevalence was based on cases ascertained by medical history only and, therefore, the true prevalence may have been underestimated. The aim of this study was to assess the prevalence of pheochromocytomas in a well-defined population of patients with hereditary head and neck paragangliomas due to a mutation in the SDHD gene. In applicable cases where the index tumour was resected, LOH for the SDHD gene in the tumours was investigated.

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Materials and methods

This study project focuses on the association of pheochromocytomas with familial head and neck paragangliomas due to germ-line mutations in the SDHD gene. For this purpose a prospective follow-up study protocol was used. We analysed all patients referred to our endocrine clinic at the Leiden University Medical Center (LUMC) with documented head and neck paragangliomas and either a proven positive family history or a proven mutation of the SDHD gene since 1 January 1988. We used a protocolized diagnostic approach aimed at detecting catecholamine excess (figure 6.1). SDHD-mutation analysis was performed as described previously.[14] These patients were part of a large cohort of patients identified with head and neck paragangliomas by the Department of Otorhinolaryngology (LUMC). There was no selection for referral based on clinical signs or symptoms compatible with pheochromocytomas or catecholamine excess or a family history of pheochromocytomas. Our center serves as a tertiary referral center for most of the western part of the Netherlands with frequent referral from regional hospitals. The treatment of hereditary paragangliomas has been centralized in Leiden.

The initial consultation included a thorough medical history, focusing on paroxysmal symptoms of palpitations, headaches, and diaphoresis. In addition, the medical and family history was evaluated and all medications were listed. All patients received a full physical examination, focusing especially on hypertension, tachycardia, and the presence of paragangliomas.

After this consultation, patients were instructed to collect urine for 24-hours on two separate occasions. Drugs known to interfere with the assays were discontinued four weeks prior to collection, or if necessary, replaced. Doxazosin (alpha-blocker) was the only anti-hypertensive drug, allowed for four weeks prior to collection of the urine. In addition, patients were instructed to follow a diet and to avoid strenuous exercise on days the 24-h samples were collected.[17] These samples were used for measurement of 24-h urinary excretion of catecholamines (epinephrine, norepinephrine, and dopamine) and vanillylmandelic acid (VMA). In order to ascertain adequacy of collection the urinary creatinine content was assessed as well.[18] Epinephrine, norepinephrine and dopamine in urine were detected by reverse-phase high pressure liquid chromatography (HPLC) with an electrochemical detector. Inter-assay coefficients of variations (CVs) for epinephrine are 3.4 % - 28.4 % ranging from high to low levels. For norepinephrine these data are 3.9 % - 7.7 % and for dopamine 3.4 % - 7.3 %. The intra-assay CVs are slightly lower.
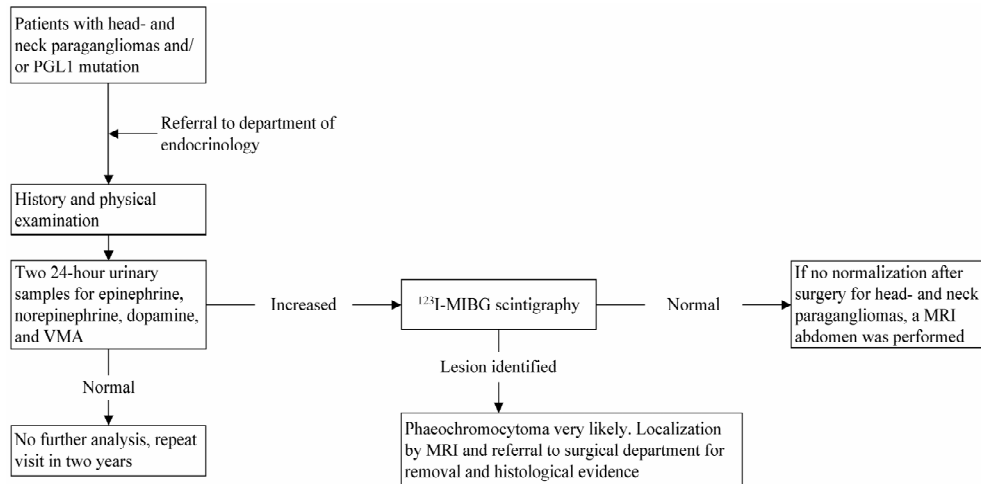
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Figure 6.1: Flowchart of protocolized endocrinological screening of referred patients with head and neck paragangliomas and/or a SDHD (PGL1) germline mutation.

VMA in urine was measured by HPLC with fluorometric detection. Here, the inter-assay CVs ranged from 2.4 to 9.1 % with also slightly lower values for the intra-assay CV.[19,20] The levels above which the 24-h urinary excretion of catecholamines was determined to be increased were: epinephrine 0.16 µmol/24 h (29µg/24h); norepinephrine 0.47 µmol/24 h (80µg/24h); dopamine 3.40 µmol/24 h (520 µg/24h); VMA 30 µmol/24 h (6 µg/24h). These cut-off values were based on studies in which 24-h urine specimens were assessed for both patients with a proven pheochromocytoma and patients referred for investigation, but subsequently found not to have a pheochromocytoma. In case only marginally elevated catecholamine levels were found, the test was repeated.[20]

Once the above-mentioned biochemical testing revealed an excess of catecholamine production, additional diagnostic tests were performed. For patients, that did not demonstrate elevated urinary levels of catecholamines or their metabolites no additional tests were ordered. After biochemical conformation of catecholamine excess had been found, [123]iodinated metaiodobenzylguanidine scintigraphy (^{123}I-MIBG) was performed. Imaging was performed 24 hours after the intravenous administration of 185 MBq of ^{123}I-MIBG (Amersham, Eindhoven, the Netherlands). Whole-body scintigraphy was done with a dual head gamma camera (Toshiba GCA 7200; Toshiba, Tokyo, Japan), which was followed by single-photon-emission computed tomography (SPECT) of the head and neck region and of the upper

abdomen. All images were analyzed visually by two experienced nuclear physicians. This strategy was chosen to avoid missing possible extra-adrenal pheochromocytomas by an initial radiological localization technique such as magnetic resonance imaging (MRI). Next to ^{123}I-MIBG scintigraphy, MRI was performed to further localize a possible pheochromocytoma. A 1.5-T whole-body scanner was used (Gyroscan ACS/NT15 and Intera; Philips, Best, the Netherlands). In case this additional radiological localization showed a lesion suspected to be a pheochromocytoma, surgery was performed, enabling a confirmation of the diagnosis by histology. During this time, analysis of the head and neck paragangliomas was performed by the department of Otorhinolaryngology. Routine follow up examinations were performed every two years.

Loss of Heterozygosity (LOH) analysis
From all patients that were operated upon for their index tumour, available tumour-specimens were retrieved from the archives of the Department of Pathology of the LUMC. Tissue specimens were archived and retrieved according to the guidelines of the Dutch Federation of Research Associations (FEDERA).
Only frozen tumour specimens were selected for DNA flow-cytometry analysis as described previously.[21] Prior to analysis selected cases were reviewed histologically and tumour percentages in the samples were determined through microscopic examination of 4μm serial sections. Additional flow sorting was performed with aneuploid and tetraploid tumours based on propidium content on a FACStar flow cytometer (Becton Dickinson, Mountain View, CA) as described.[9] A minimum of 10.000 nuclei were sorted in microfuge tubes. From available peripheral blood samples, diploid (unsorted) tumours, and sorted aneuploid cases, subsequent DNA isolation was performed as described.[22] Isolated DNA was screened for the Dutch SDHD germ-line mutation Asp92Tyr (D92Y) by restriction digestion as described.[14] The presence of SDHD mutations and allelic imbalance (retention or loss) of the wild type gene was determined through examination of digestion patterns. LOH was considered present when the ratio between mutant and wild type alleles was larger than 2, according to restriction intensity measurements. DNA extracted from peripheral blood samples from a patient with confirmed mutations and a non SDHD-linked patient served as control-samples to validate the PCR-process and restriction digestion analysis.

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The software package used for statistical analysis was SPSS for Windows 10.0.5.[23] The results of statistical tests were considered significant at a level of p-value < 0.05.

Results

Between 1988 and 2003, 40 consecutive patients (20 male and 20 female) with documented head and neck paragangliomas were referred to our endocrine clinic for a protocolized endocrinological screening. The patient characteristics are summarized in Table 6.1. All but one reported familial cases of head- and neck paragangliomas, whereas the SDHD gene mutation was ascertained in 26 individuals (25 with D92Y and 1 with Leu139Pro mutation), and documented to be present within a family member in another seven cases. The one patient without a positive family history for head and neck paragangliomas was found positive for the SDHD gene mutation. The total patient population included members from 24 separate families.

Half of the patients observed in this study were found to have multiple head and neck paragangliomas. The most common types of paraganglioma were carotid body tumours (85%) and vagal nerve tumours (47.5%; Table 6.1).

A thorough medical history revealed actual paroxysmal symptoms of palpitations, headaches, and diaphoresis in 12 patients (30%). Furthermore, 10 patients were previously diagnosed with hypertension and 7 of those 10 patients were actually using anti-hypertensive medication. During physical examination another 16 patients were found to have a blood pressure greater than 140/90 mmHg.

The results of the protocolized screening of the patients are shown in Figure 6.2. After collection of two 24-hour urinary samples 17 patients (42.5%) were categorized as having catecholamine excess. The urinary tests were normal in 23 patients.

All, but three, patients identified with increased catecholamine excretion were analyzed by ^{123}I-MIBG scintigraphy. In two of these three patients the first measurement yielded only slightly increased values (single norepinephrine measurements of 0.49 and 0.51 μg/24h) and repeated measurements revealed normal excretion of catechoalmines. In a third patient catecholamine levels were found to have normalized after removal of the head and neck paragangliomas. The remaining 14 patients with an increased 24-hour urinary catecholamine levels underwent ^{123}I-MIBG scintigraphy.

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	Number (percentage)
Age (mean)	45.8 years (SD ±11.8)
Gender	Male: n = 20; Female: n = 20
SDHD gene mutation ascertained in case	n = 26 (65.0%)
SDHD gene mutation ascertained in family	n = 7 (17.5%)
Familial head- and neck paragangliomas	n = 39 (97.5%)
Types of head- and neck paragangliomas	
- Glomus Caroticum	n = 34 (85.0%)
- Glomus Vagale	n = 19 (47.5%)
- Glomus Jugulotympanicum	n = 13 (32.5%)
Multiple head- and neck paragangliomas	n = 20 (50%)
Follow up duration (median)	5.7 years (SD ± 7.8, median 2)
Paroxysmal symptoms	12 (30%)
History of hypertension	10 (25%)
Hypertension	26 (65%)

Table 6.1: Patients' characteristics (n = 40)

The results of ^{123}I-MIBG scintigraphy showed a normal distribution in 5 patients. Four of these patients were found to have no abdominal lesions on MRI suggestive of pheochromocytoma. Three of the four patients analyzed by MRI of the abdomen previously underwent (subtotal) surgery for head and neck paragangliomas (one with bilateral paragangliomas was operated on only unilaterally), but postoperatively continued to show an increased 24-hour urinary excretion of catecholamines. Another patient had normalization of the 24-hour urinary samples after surgery.

 In nine patients with catecholamine excess a lesion on ^{123}I-MIBG scintigraphy was found in the abdomen. MRI-investigation revealed a pheochromocytoma in seven patients, which was confirmed histopathologically after surgical removal of the lesion. One patient was discovered with an extra-adrenal paraganglioma. In only one patient MRI scanning failed to reveal a pheochromocytoma. In this particular patient the increased catecholamine was dopamine.

Thus, in 15 of the 40 patients included (37.5%), sustained urinary catecholamine excess was detected. In six of these 15 patients ^{123}I-MIBG scintigraphy and MRI abdomen could not demonstrate a pheochromocytoma and in one patient the excess did subside after removal of the head and neck paragangliomas after which no further tests were performed. This indicates that head and neck paragangliomas may be responsible for excess catecholamine production in about 17.5 % of the patients included in the study (n=7). Although quantitative assessment of
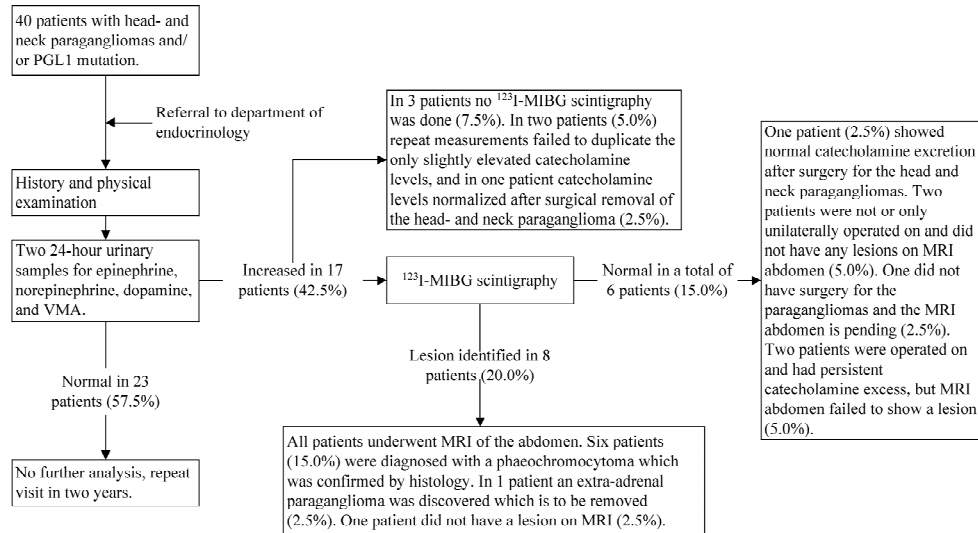
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Figure 6.2: Flowchart with results of work-up of referred patients with head and neck paragangliomas and/or a SDHD (PGL1) germline mutation.

catecholamine secretion in 24 hour urine samples has a specificity between 69 and 95%, it seems unlikely that false positive samples explain the persistent excess catecholamine production.[24] Another eight patients (20%) were diagnosed with a pheochromocytoma, including one patient with an extra-adrenal tumour.

In an univariate analysis no distinctive parameters could be demonstrated identifiyng either the 15 patients with catecholamine excess or the eight patients that were diagnosed with (extra-adrenal) pheochromocytoma (Table 6.2). However, there was a striking, but not significant, difference in follow-up duration between patients with and without the presence of a pheochromocytoma (10.4 ± 9.3 S.D. versus 6.4 ± 7.1 S.D. years). Indeed, some patients were diagnosed with head and neck paragangliomas early, allowing more biennial visits for screening of catecholamine excess.

Parameter	Catecholamine excess		Phaeochromocytomas	
	Present (n=15)	Absent (n=25)	Present (n=7)	Absent (n=33)
Age	45.0 (SD ± 13.1)	46.3 (SD ± 11.2)	48.5 (SD ± 12.9)	45.3 (SD ± 11.7)
Gender (m/f)	9 / 6	11 / 14	4 / 3	16 / 17
Duration of follow-up	6.6 (SD ± 8.5, median 3)	5.1 (SD ± 7.5, median 2)	10.2 (SD ± 9.9, median 8)	4.7 (SD ± 7.2, median 1)
Paroxysmal symptoms	4 (26.7%)	8 (32.0%)	1 (14.3)	11 (33.3%)
History of hypertension	3 (20%)	7 (28.0%)	2 (28.6%)	8 (24.2%)
Hypertension	11 (73.3%)	15 (60.0%)	5 (71.4%)	21 (63.6%)
Type of head- and neck paraganglioma				
Caroticum	12 (80.8%)	22 (88.0%)	6 (85.7%)	28 (84.8%)
Vagale	6 (40.0%)	13 (52.0%)	3 (42.9%)	16 (48.5%)
Jugulotympanicum	5 (33.3%)	8 (32.0%)	2 (28.6%)	11 (33.3%)

Table 6.2: Univariate analysis of possible distinctive parameters for catecholamine excess or pheochromocytomas.

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LOH analysis:

Suitable frozen tumour specimens were retrieved from four of 15 patients with catecholamine excess that were detected after screening. The selected specimens included 2 adrenal pheochromocytomas, one extra adrenal pheochromocytoma and one jugulotympanic paraganglioma. Additionally, one extra-adrenal pheochromocytoma was retrieved from a member of a PGL1 family that was not included in the initial patient-selection (tumor 5). The clinicopathological characteristics of the tumours are listed in Table 6.3. All tumors were clinically benign. Histologically the pheochromocytomas displayed proliferations of chief cells embedded in a highly vascularised stroma. The jugulotympanic tumour was organized in a typical Zellballen-pattern with clusters of chief cells and sustentacular cells located at the periphery. Flow cytometric analysis revealed aneuploid populations in 3 tumours and tetraploidy in two tumours. From the aneuploid and tetraploid tumours, different cell populations were subsequently isolated by flow sorting based on their DNA content.

Mutation analysis of peripheral blood samples revealed the D92Y founder mutation in exon 3 of the SDHD-gene in all cases. Subsequent restriction-digestion analysis of peripheral blood samples and sorted tumor fractions displayed LOH of the wild type SDHD-allele in all tumours examined (Figure 6.3).

Tumor	Location	Ploidy	Tumor-%	Mutation	r-d analysis
1	JTT	a	70%	D92Y	LOH
2	eap	t	70%	D92Y	LOH
3	phaeo	a	70-100%	D92Y	LOH
4	phaeo	a	40%	D92Y	LOH
5	eap	t	60%	D92Y	LOH

Table 6.3: Clinicopathological characteristics of tumors selected for LOH-analysis: *JJT, phaeo, eap*: jugulotympanic tumor, phaeochromocytoma, extra-adrenal phaeochromocytoma, *a,t*: aneuploid, tetraploid, *D92Y*: SDHD germline-mutation exon 3, *r-d analysis*: restriction-digestion analysis (see text), *LOH*: loss of heterozygosity (see text).
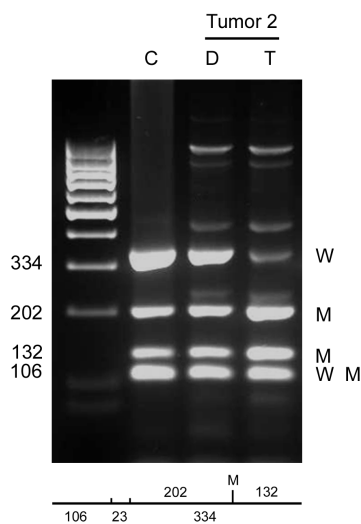
Figure 6.3: Loss of wild-type SDHD allele in sorted tumor fraction from an extra-adrenal pheochromocytoma. After PCR amplification of exon 3, digestion was performed with restriction enzyme *RsaI* to detect the Asp92Tyr mutation. The wild-type allele contains two restriction sites that will result in three fragments of 23, 106 and 334 bp after digestion (the smallest is not detectable). The mutation creates a third restriction site within the 334 bp fragment resulting in two additional fragments of 202 and 132 bp. The digest from the diploid population shows a wild-type and mutant allele. In the sorted tumour fraction, loss of the wild-type allele is observed. LOH occurs in this fraction since the ratio between the mutant and wild-type alleles is larger than 2 according to restriction-fragment intensity measurements (see text). *C*: control digestion obtained from a peripheral blood sample with confirmed Asp92Tyr mutation. *D*: normal (diploid) DNA obtained from peripheral blood sample of patient. *T*: DNA from sorted tetraploid tumor fraction. *W* and *M*: wild-type and mutant fragments. In some fractions additional fragments are present due to incomplete digestion.

Discussion

This is the first study specifically addressing the prevalence of catecholamine excess and pheochromocytomas in a well-defined population with head and neck paragangliomas. In 37.5% of the examined patients, the catecholamine levels were elevated and a pheochromocytoma was detected in 20% of the patients. Therefore, this high prevelance of catecholamine excess and pheochromocytomas is in contrast with those in previous observations.[13,25-27] Furthermore, it is likely that the increased prevalence of pheochromocytomas documented in the present study is a minimal estimate. As the protocol is repeated every 2 years more patients with catecholamine excess are expected to arise. Therefore, it is likely that longer duration of follow up will also reveal a higher prevalence of pheochromocytomas. We studied a specific population of Dutch patients with head and neck paragangliomas due to a SDHD mutation, as the prevalence of catecholamine in these patients was suspected to ascend above the previously mentioned prevalence of about 5 percent.[13] In our study 82.5 % of patients were found to have a positive family history for head and neck paraglioma and/or were diagnosed with a SDHD gene mutation. The remaining 17.5 % only had a positive family history for head and neck paraglioma and refused genetic testing for different reasons. Since SDHD mutations explain the inheritance of head and neck paraglioma in 97% percent of Dutch families, we did not exclude these patients

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from our analysis.[14] In addition, we found that in the seven patients without ascertainment of the SDHD mutation only two had catecholamine excess and none were diagnosed with a pheochromocytoma. Therefore, excluding these patients from the analysis will only strengthen the association between hereditary head and neck paragangliomas with a proven SDHD mutation and the prevalence of pheochromocytomas.

Subsequent genetic analysis of extirpated tumours revealed LOH of the wild-type SDHD-gene in all investigated cases. This finding strongly implicates a role for SDHD as a tumoursuppressor gene in the tumourigenesis of pheochromocytoma as has been described previously in the development of head and neck paragangliomas.[9,28] However, due to the low prevalence of pheochromocytomas in relation to the prevalence of head and neck paragangliomas in families with SDHD gene mutations, one may hypothesize that additional mutations are required for the development of meta- or synchronous pheochromocytomas in addition to head and neck paragangliomas.[29]

A protocolized diagnostic strategy was used to evaluate all patients referred to the endocrinology clinic with documented head and neck paragangliomas and either a positive family history for paragangliomas or a proven SDHD gene mutation. Patients were referred to the endocrinology department for routine consultation, irrespective of the results of medical history and physical examination by the referring physician. This is further demonstrated by the absence of a clear correlation between catecholamine excess and presenting signs and symptoms. The longer follow-up time of patients diagnosed with a pheochromocytoma cannot therefore be explained merely by referral bias. A possible explanation for the observed difference in follow-up may be the time needed for patients to develop a pheochromocytoma during their life. Possibly, head and neck paragangliomas are detected earlier in life as they are located superficially, whereas pheochromocytomas are not detectable during physical examination and further analysis through urine sampling is needed to obtain the correct diagnosis. Furthermore, as mentioned above, additional mutations may be required for a patient to develop pheochromocytomas in addition to head and neck paragangliomas.

Although current standards for biochemical diagnosis of a pheochromocytoma may dictate a biochemical screening for suspected cases of pheochromocytomas with plasma free metanephrine and urinary metanephrine levels, these tests are currently unavailable in our laboratory. In particular, plasma free metanephrines

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have shown sensitivity and specificity of 97% and 96%, respectively, in hereditary pheochromocytomas.[24,30] However, urinary free catecholamines were found to have a sensitivity of more than 90%.[4,31] Although VMA measurement in urine samples demonstrates a low sensitivity, the specificity is very high with 99% among hereditary cases of pheochromocytomas.[24]

We used a temporized approach, starting with the measurement of urinary free catecholamines and VMA. This biochemical evaluation was then followed by MIBG scintigraphy and MRI of the abdomen, allowing for adequate diagnosis. Urinary sampling confirmed the diagnosis of catecholamine excess, whereas anatomical identification of (extra) adrenal pheochromocytomas was achieved by MIBG-scintigraphy and MRI. Especially in hereditary pheochromocytomas, MIBG scintigraphy has a high specificity, almost 100%, with a sensitivity approximating 90%, whereas MRI scanning has a higher sensitivity of almost 100%, in particular for extra-adrenal pheochromocytomas.[32-34] Combination of both diagnostic techniques yields the most information. Since the strategy used in this protocol for biochemical testing may be less sensitive we might have underestimated the true prevalence of catecholamine excess. The one patient that was identified as having an increased urinary excretion of dopamine was later found to have a positive MIBG scan, but a negative MRI. A possible explanation may be the fact that dopamine is less specific in detecting pheochromocytomas, as it is found to be more widespread in the body. An accumulation of dopamine will then appear on MIBG scanning, but not on MRI, as there is no actual pheochromocytoma. In addition, we cannot exclude the possibility of the presence of adrenal tumours in the patients with negative biochemical screening of catecholamine excess, since we did not perform visualization studies in those patients. However, this possibility does not invalidate our conclusions with respect to the high prevalence of pheochromocytomas.

Remarkably, the likelihood of catecholamine excess cannot be predicted by signs and symptoms. This observation is in concordance with previous reports in the literature. Generally pheochromocytomas are described as a distinct clinical entity, but in clinical practice the symptoms may be misleading or even absent, as is known in other familial syndromes with pheochromocytomas such as multiple endocrine neoplasia type 2, VHL-disease, and neurofibromatosis 1.[30] Apparently, pheochromocytomas are detected in an earlier phase in those genetic syndromes by biochemical screening, prior to development of the classical signs and symptoms of pheochromocytomas. Therefore, in these groups of patients biochemical testing

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is obligatory. The warning symptoms of catecholamine excess may frequently be lacking, because the observed prevalence of pheochromocytomas at autopsies is increased almost 500- fold compared to the prevalence in the general living population.[35,36] In accordance, even in our study, which focused on the detection of pheochromocytomas, patients experienced hardly any signs or symptoms classically associated with pheochromocytomas. Therefore in many cases pheochromocytomas may not be detected merely from signs and symptoms.

The increased prevalence of catecholamine excess and pheochromocytomas has grave consequences for the future follow-up of patients with head and neck paragangliomas with a documented or suspected SDHD mutation. Catecholamine excess may implicate special pre-operative screening in order to minimize hazards of anesthesia and surgery for head and neck paragangliomas or other procedures. Sudden changes in blood pressure and electrocardiographic patterns might be prevented if patients are adequately screened pre-operatively for catecholamine excess. Moreover, the question may be raised whether an increased prevalence of catecholamine excess may be present in asymptomatic carriers of the SDHD gene mutation, *i.e.* without head and neck paragangliomas. This needs to be addressed in future studies.

In conclusion, the prevalence of catecholamine excess and pheochromocytomas in a well-defined Dutch population with hereditary head and neck paragangliomas due to a germ-line mutation in the SDHD gene is remarkably high. The loss of the wild-type SDHD allele in the pheochromocytomas from patients with germline SDHD mutations strongly supports the evidence that these tumors belong to the SDHD-associated tumour spectrum.

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Chapter 7

Summary and concluding remarks

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Summary

Head and Neck Paragangliomas (HNP) are hypervascular tumours characterised by a slow growth pattern and a strong hereditary context. The tumours originate from the neural crest derived paraganglia, which are associated with the autonomous nervous system and are situated at several locations in the head and neck region. Treatment strategies for these tumours have remained controversial. Especially in case of skull base tumours, radical surgical resection is often difficult to achieve and can be associated with complications such as peroperative haemorrhage or postoperative cranial nerve damage that result in considerable morbidity and loss of quality of life (cranial nerve palsy, CVA). These considerations are even more pertinent /important in hereditary cases, in which the patients often develop multiple tumours that would require several surgical procedures. Radiation therapy is being advocated and several studies have reported successful control of the disease. However these results may be attributable to the general slow growth of the tumours as most studies lack long-term follow-up of the patients. Because of the indolent growth pattern and benign behaviour of HNP, conservative approaches have become important alternatives in the treatment strategy. This is especially relevant in patients with multiple tumours where debilitating surgery could result in double sided cranial nerve palsy. Generally, in cases of vagal nerve tumours or glomus jugulare tumours, a policy of watchful monitoring & waiting is propagated, only intervening when obvious tumour progression or clinical deterioration is apparent.

Associated with the natural course of HNP is the strong hereditary context of these tumours. The identification of SDHD as susceptibility gene in hereditary HNP has lead to a novel concept of mitochondrial tumoursuppressor-genes and further insight in the intricate association of cellular oxygen sensing mechanisms and (pseudo)-hypoxia as environmental risk factors in the development of these tumours as well as pheochromocytomas. However, further characterisation of the tumour biology is warranted for better understanding of the natural behaviour of HNP and possible identification of clinicopathological parameters that could aid the clinician in his treatment decisions. Additionally, the relationship between SDH-linked HNP and the development of pheochromocytomas requires further investigation as the latter tumours can pose endocrinological threats as well as increased danger of malignant disease.

This thesis has focussed on the molecular pathology of HNP in order to gain further

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insight in intrinsic characteristics of these tumours. Additionally, the relation of HNP and pheochromocytomas was further investigated in cooperation with the Department of Endocrinology.

Chapter 1 reviews the literature concerning HNP and contains general data about the disease. In the later paragraphs of this chapter the current concepts regarding molecular genetics and tumourigenesis in HNP are discussed with special reference to the tumoursuppresive nature of SDH genes and possible pathways that may be involved in tumour development.

The discovery of mutations in genes encoding for subunits of complex II of the mitochondrial respiratory chain (SDH) in hereditary paragangliomas has lead to new insights in the development of HNP. The phenotypic consequences of these gene mutations are slowly becoming understood. In **chapter 2** the SDH enzyme activity was studied in 22 SDHD-linked and sporadic HNP using an enzyme-histochemical technique.[1] As expected, in all SDHD-linked tumours SDH-activity was absent in the tumour-cells, demonstrating that SDHD-mutations indeed affect the function of complex II. Moreover SDH-activity was also reduced in the majority of sporadic cases, indicating that perturbed complex II function is generally involved in the development of HNP, irrespective of an underlying hereditary defect in one of the subunits. The loss of SDH-activity was found to be associated with aberrant expression of the two catalytic subunits of complex II in an immunohistochemical experiment. Immunoelectronmicroscopy showed disturbed mitochondrial morphology and confirmed the aberrant expression of these subunits within mitochondria. In most of the tumours studied, the flavoprotein subunit (encoded by SDHA) showed elevated expression whereas the expression of the ironprotein subunit (encoded by SDHB) was generally reduced. However, in two sporadic cases SDH-activity was present in the tumour cells with concomitant immunohistochemical expression of the ironprotein. These findings suggest that the reduced expression of the ironprotein may be a direct consequence of prior events such as gene mutations in one of the anchor-subunits (SDHC and SDHD) and that the elevated expression of the flavoprotein may be regarded as an attempt to compensate for functional loss. This hypothesis is supported by the observation that SDHB gene mutations are also involved in hereditary paragangliomas and pheochromocytomas whereas SDHA-mutations cause a completely different disease-entity: *Leigh's syndrome*. This syndrome is characterised by progressive

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neurodegenerative disease with clinical features such as optical atrophy, ataxia and myopathy but without predisposition for development of paragangliomas.[2,3] Interestingly, the two sporadic cases that retained SDH-activity and ironprotein-expression indicate that other mechanisms than complex II dysfunction may be operational in the development of paraglioma.

Recent studies on phenotypic consequences of SDHD and SDHB-gene mutations have focussed on HIF associated pathways and report elevated expression of VEGF in tumours. However, as has been pointed out by several authors, this pathway probably does not fully account for tumour development in HNP and pheochromocytomas. In **chapter 3** the role of the angiogenic growth factor bFGF was examined. The immunohistochemical expression of bFGF and its high affinity receptor FGFR1 was studied in 7 carotid bodies and 33 HNP. bFGF was present in both carotid bodies and tumours confirming earlier reports. FGFR1 was moderately present in normal carotid bodies but the expression was increased in tumours possibly in response to (pseudo) hypoxic conditions. The increase of FGFR1-expression and the simultaneous presence of the ligand bFGF could indicate the existence of an autocrine or paracrine mechanism that may present an alternative pathway in the development of HNP[4]. Not only could bFGF impose a mitogenic signal, its actions may also enhance HIF-mediated pathways such as VEGF or increase apoptotic resistance through upregulation of anti-apoptotic Bcl-proteins.

In **chapter 4** proliferation, apoptosis and aspects of the cell cycle were investigated to further characterise the molecular events that determine the extraordinary indolent growth pattern of HNP.[5] In a series of 42 SDHD-linked and sporadic HNP an immunohistochemical study was performed with the proliferation marker Ki-67, cell cycle proteins p53 and p21^{WAF}, and the anti-apoptotic protein Bcl-x$_L$. The presence of apoptosis was investigated with a TUNEL assay in a subset of 10 tumours. Additionally, the DNA-ploidy of the tumours was determined with flow cytometry to examine the presence of DNA aberrations and signs of DNA synthesis, which may be indicative of possible cell cycle activity. The average proliferation index as calculated with Ki-67 was approximately 1% and is in accordance with the clinical slow growth of the tumours. The expression profile of p53 was not indicative for mutations of this important regulator of cell cycle control whereas the downstream cell cycle inhibitor p21^{WAF} was diffusely expressed among tumour cells. Flow cytometry showed DNA-aberrations in approximately 50 % of the

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tumours with a considerable percentage of cases with tetraploidy or a high G_2/M phase. These combined data suggest the presence of a small number of cycling cells with activation of cell cycle checkpoints that may have resulted in a G_2M arrest. Apparently there is no strong selection pressure on relieving checkpoints that restrain enhanced proliferation in HNP. A possible explanation for such low selection pressure would be blocked apoptosis that prevents checkpoint-induced elimination. The TUNEL assay showed no signs of apoptosis suggesting that apoptotic activity is low or inhibited by the tumour cells. Lack of apoptosis could be due to expression of the anti-apoptotic Bcl-protein Bcl-x$_L$ that we observed in our series. Interestingly, several studies mention similar modes of action, in which expression of Bcl-x$_L$ may overcome a p53 induced G_2M cell cycle arrest allowing arrested cells to escape and continue to proliferate, eventually leading to polyploidisation and tetraploidy.[6-8]

Thus a combination of a weak mitogenic stimulus, functional cell cycle checkpoints and inhibited apoptosis could lead to a small fraction of proliferating tumour cells in which malignant degeneration is attenuated but allowing tumour cells to survive. Such a model could explain a slow growing tumour with a relative indolent behaviour as is generally observed in HNP.

A striking histological feature of paragangliomas is the frequent organisation of the tumour in *Zellballen* with chief cells and sustentacular cells that closely resembles the architecture of the normal paraganglion-tissue. Although there is general consensus that chief cells represent the major neoplastic component in HNP, the nature of sustentacular cells has remained obscure. The origin of sustentacular cells was investigated in **chapter 5**, using multi-parameter flow cytometry on 6 HNP.[9] In this experiment the sustentacular cell-fraction was identified by labelling with S-100 and was found to be consistently diploid in aneuploid tumours. After cell sorting, the S-100 labelled cell fractions showed retention of the wild type SDHD-allele in contrast to the loss of heterozygosity in the aneuploid fractions in 3 SDHD-linked cases. These results indicate that sustentacular cells do not belong to the tumour cell population and are probably derived from stromal tissue. Chief cells thus represent the only neoplastic component in paragangliomas.

Apart from hereditary HNP, the spectrum of SDHD mutations also involves the development of pheochromocytomas. The prevalence of synchronic or metachronic pheochromocytomas among patients with confirmed SDHD-linked HNP was

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investigated in cooperation with the department Endocrinology. In **chapter 6**, the results of this study are presented.[10] In a series of 40 patients referred with SDHD-linked HNP, 15 patients were found to have elevated catecholamine excess and in 9 of these patients (20% of all patients) a pheochromocytoma was detected. In a subset of extirpated pheochromocytomas, loss of heterozygosity of the SDHD wild type allele confirmed the association of SDHD mutations with the development of these tumours.

Conclusion

This thesis has focussed on the molecular biology and clinicopathological aspects of HNP and further characterized the events that lead to the development and behaviour of these tumours.

The recently discovered mutations in genes of subunits of complex II have created new insights in the molecular genetic events and the role of these tumoursuppressor genes in HNP. SDH-dysfunction and destabilisation of complex II is present in SDHD-linked HNP but also in the majority of sporadic tumours and possibly is a common event in the development of paragangliomas in general.

Recent studies on downstream pathways have shown the activation of HIF and expression of VEGF in tumours. However a paracrine or autocrine mechanism involving the angiogenic growth factor bFGF and the high affinity receptor FGFR1 may also contribute to tumour development in paragangliomas. Apart from mitogenic signalling pathways, tumourigenesis was also investigated at the level of the cell cycle and the proliferation-index of the tumours was determined. Based on the results, a model is presented in which the combined effects of a weak mitogenic stimulus, active cell cycle control with induction of cell cycle arrest, and lack or inhibited apoptosis lead to a slowly proliferating population of tumour cells with progressive polyploidisation that has a low propensity for further (malignant) degeneration. (Chapter 4, figure 4.4)[4] Further investigations will be required to confirm these events and may eventually lead to parameters that can predict the clinical behaviour of paragangliomas in the future.

In a separate study, the controversy regarding the nature of sustentacular cells in HNP has been resolved by demonstrating that this cell fraction does not belong to the tumour population and is presumably of stromal origin. Consequently, paragangliomas should be regarded as a monoclonal proliferation of chief cells without biphasic properties.

Finally, in cooperation with the department of Endocrinology it has been demonstrated that a considerable number of patients with SDHD-linked HNP also develop pheochromocytomas. This finding warrants standard endocrinological examination of patients with SDHD-linked HNP. Further studies will be required to establish the penetrance of SDHD-linked pheochromocytomas and to determine the frequency of endocrinological screening for these tumours in the future.

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Samenvatting

Paragangliomen zijn zeldzame vaatrijke tumoren die ontstaan uit paraganglia, kleine orgaantjes die gegroepeerd zijn rond de lange banen van het autonome zenuwstelsel. Er worden twee verschillende typen paraganglia onderscheiden: het eerste type is geassocieerd met het sympathische deel van het autonome zenuwstelsel en de paraganglia hiervan zijn gelokaliseerd in de borstholte en buik-holte met als voornaamste station het bijniermerg. Tumoren die uit deze paraganglia of bijniermerg ontstaan zijn vaak hormonaal actief en worden vaak aangeduid als feochromocytoom. Het tweede type paraganglia is geassocieerd met het parasympathische deel van het autonome zenuwstelsel en is voornamelijk gele-gen in het hoofd hals gebied. Het voornaamste paraganglion is het carotid body dat is gelegen in de vork van de grote halsslagader (arteria carotis). Het functio-neert als een chemoreceptor en registreert de zuurstofspanning in het bloed en is verbonden met het ademhalingscentrum in de hersenstam. Tumoren die uit hoofd-hals-paraganglia ontstaan, staan bekend als hoofd-hals-paragangliomen (syn.: glomus tumor of chemodectoom). Zij vertonen in de regel een indolent gedrag en groeien in het algemeen zeer langzaam. Vanwege hun kritische lokalisatie dichtbij slagaders en hersenzenuwen kunnen zij echter aanzienlijke morbiditeit veroorza-ken (vnl. hersenzenuwuitval). Bovendien kan in uitzonderingsgevallen snelle groei en uitzaaiing wel degelijk optreden.

De ontwikkeling van hoofd-hals-paragangliomen is vaak erfelijk bepaald waarbij de overerving een bijzondere eigenschap vertoont: alleen als de erfelijke eigen-schap wordt doorgegeven via een mannelijke drager zal bij het nageslacht tumor-vorming optreden. Overdracht via een vrouw leidt nooit tot tumorvorming bij haar kinderen maar zij blijven wel drager van de aandoening. Deze geslachtsspecifieke overerving wordt aangeduid als genomische imprinting. Bovendien ontwikkelen patiënten met erfelijke paragangliomen vaak tumoren op meerdere locaties en doorgaans op jongere leeftijd.

Recent zijn via genetisch familieonderzoek de verantwoordelijke mutaties geïden-tificeerd die betrokken zijn bij het ontstaan van erfelijke paragangliomen. Het betreffen mutaties in genen van complex II (syn: succinaat dehydrogenase; *SDH*) dat een onderdeel vormt van de intracellulaire ademhalingsketen die zich bevindt in kleine celorganellen: de mitochondria. Vermoedelijk verstoren de mutaties de functie van complex II en daarmee de intracellulaire zuurstof registratie in paraganglion-cellen.

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Welke mechanismen vervolgens verder betrokken zijn in het ontstaan van tumoren is nog onvoldoende bekend, maar er zijn aanwijzingen dat mogelijk een pseudo-registratie van zuurstofgebrek (pseudo-hypoxie) signaalroutes activeert die cellen stimuleren tot celdeling.

Behandeling van hoofd-hals-paragangliomen is in de regel chirurgisch. Dit is in sommige gevallen geen gemakkelijke opgave vanwege de innige relatie van de tumor met bloedvaten en hersenzenuwen en slecht toegankelijke operatiegebieden zoals de schedelbasis. Chirurgische verwijdering kan hierdoor worden gecompliceerd door bloeding of hersenzenuwuitval. Daarnaast is bij erfelijke patiënten regelmatig sprake van meerdere tumoren of is er verhoogd risico op ontwikkeling van nieuwe tumoren, zodat bij deze patiënten soms risico bestaat op dubbelzijdige hersenzenuwuitval (al of niet tgv chirurgische complicaties). Het natuurlijke beloop van hoofd-hals-paragangliomen is daarentegen in veel gevallen mild: de tumoren groeien zeer langzaam en geven lange tijd weinig klachten. Vanwege de complicatie risico's en het dilemma van multipele tumoren wordt daarom regelmatig een afwachtend beleid gevoerd waarbij periodieke controles plaatsvinden (wait & scan) en alleen wordt besloten tot chirurgie bij toename van klachten of evidente groei (klinisch of radiologisch) van de tumor.

Ondanks de identificatie van de eerder genoemde kiembaanmutaties bij erfelijke tumoren is tot heden nog weinig bekend van de tumorbiologie van hoofd-hals-paragangliomen. Er zijn derhalve nog geen betrouwbare parameters beschikbaar waarmee de behandelend clinicus het natuurlijk gedrag van individuele tumoren kan voorspellen wat weer van belang kan zijn voor de behandelingsstrategie.

Dit proefschrift beschrijft een aantal studies waarin moleculair-pathologische aspecten van hoofd-hals-paragangliomen nader zijn bestudeerd teneinde de tumor biologie verder op te helderen.

In **Hoofdstuk 1** wordt een beknopt overzicht gepresenteerd van de huidige kennis en literatuur over het paraganglion-systeem en hoofd-hals-paragangliomen met focus op tumorbiologische aspecten.

De effecten van SDH-mutaties en de rol van complex II in de tumorvorming van hoofd-hals-paragangliomen zijn nader onderzocht in **Hoofdstuk 2**. In 14 erfelijke paragangliomen en 8 sporadische tumoren is de SDH-activiteit bepaald met behulp van enzymhistochemie. Daarnaast werd in de tumoren de expressie van de twee enzymatische subunits van complex II, het flavoprotein (gecodeerd door

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SDHA) en het ironprotein (gecodeerd door SDHB), mbv immunohistochemie on-
derzocht. In een aantal tumoren werd het morfologische aspect van de mitochondria
en de locatie van subunits met behulp van elektronenmicroscopie bestudeerd.
SDH-activiteit was afwezig in alle erfelijke tumoren zodat kan worden geconclu-
deerd dat SDH mutaties leiden tot afgenomen enzymactiviteit. Bovendien kon
ook in het merendeel van de sporadische tumoren geen activiteit in de tumor-
cellen worden gedetecteerd, wat erop duidt dat SDH-inactivatie kennelijk een
algemeen verschijnsel is bij het ontstaan van paragangliomen. Verlies van SDH-
activiteit lijkt daarbij samen te gaan met verminderde expressie van het ironprotein
en er is sprake van een toename van het aantal mitochondria met een gezwollen
aspect. Er blijken echter ook sporadische tumoren te zijn die dit patroon niet
volgen en wel degelijk SDH-activiteit vertonen in combinatie met normale expres-
sie van ironprotein. Hieruit kan worden afgeleid dat kennelijk ook andere mecha-
nismen betrokken zijn bij tumorvorming.

De betrokkenheid van complex II-genen bij erfelijke paragangliomen heeft geleid
tot nieuwe inzichten in de rol van de mitochondriale ademhalingsketen bij tumor-
vorming. Over de wijze waardoor het verminderd functioneren van complex II tot
proliferatie en tumorvorming leidt, bestaan verschillende hypothesen. Vermoede-
lijk ontstaan door disfunctie van de ademhalingsketen reactieve zuurstofvormen
(reactive oxygen species) of pseudo-hypoxie in de chief cellen van paraganglia
waarop een cascade van signalen wordt geïnitieerd die leidt tot activatie van fac-
toren die cellen aanzet tot proliferatie. Een belangrijk signaal eiwit is vermoedelijk
HIF 1a (hypoxia inducible factor 1a) dat een centrale rol lijkt te spelen in de
respons van cellen bij hypoxie. Echter niet alle vormen van proliferatie lijken vol-
ledig door activatie van HIF 1a te kunnen worden verklaard. Een tweede factor
waarvan bekend is dat het vaatnieuwvorming bij hypoxie stimuleert is bFGF (ba-
sic fibroblast growth factor). Bovendien blijkt deze factor in eerdere experimen-
ten met gekweekte "chief cellen" van carotid bodies proliferatie en overleving te
stimuleren onder zuurstofarme condities.
De expressie van bFGF en zijn receptor FGFR1 (fibroblastic growth factor receptor
1) is onderzocht in een serie van 7 normale carotid bodies en 34 paragangliomen
door middel van immunohistochemie. De resultaten van deze experimenten wor-
den besproken in **Hoofdstuk 3**. bFGF komt zowel in de carotid bodies als de
tumoren sterk tot expressie. De receptor FGFR1 is aanwezig in normale carotid
bodies maar komt versterkt tot expressie in de tumoren. Mogelijk is deze ver-

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hoogde expressie een reactie op (pseudo) hypoxische omstandigheden in de chief cellen en leidt dit tot een verhoogde gevoeligheid van de cellen voor het aanwezige bFGF. Binding van bFGF zou vervolgens de cellen kunnen stimuleren tot proliferatie of de cellen ongevoeliger kunnen maken voor geprogrammeerde celdood (apoptose).

Hoe een proliferatief signaal vervolgens leidt tot uiteindelijke tumorvorming in paragangliomen is goeddeels onbekend. Een klinisch belangrijk aspect van hoofd-hals-paragangliomen is daarbij de doorgaans uitzonderlijk langzame groei en het indolente gedrag van de tumoren. Verdere karakterisering van de processen die deze langzame groei bepalen kan in de toekomst leiden tot nieuwe parameters die het gedrag van tumoren voorspellen. Voor proliferatie en tumorgroei dienen tumorcellen zich te vermenigvuldigen via celdeling (mitose). Voorafgaand aan mitose dienen cellen de cel cyclus te doorlopen waarin verschillende checkpoints aanwezig zijn waar de voorbereiding van het delingsproces wordt gecontroleerd. Fouten en onregelmatigheden tijdens de cel cyclus kunnen leiden tot een arrest in een checkpoint of tot apoptose. Op deze wijze worden delingsfouten voorkomen die mogelijk leiden tot disfunctie of autonome groei. Veel tumoren zijn in staat om deze checkpoints te omzeilen of blijken ongevoelig voor apoptose.

In **Hoofdstuk 4** zijn verschillende aspecten van de cel cyclus, proliferatie, en apoptose onderzocht in 42 erfelijke en sporadische hoofd-hals-paragangliomen. De mate van proliferatie werd bepaald door meting van het aantal prolifererende cellen met de marker Ki-67 waaruit een proliferatie-index werd berekend. Deze bleek voor paragangliomen laag te zijn ($\sim$1%) en sluit goed aan bij de klinisch langzame groei. De expressie van een aantal factoren dat een rol speelt in de cel cyclus en apoptose werd bestudeerd met behulp van immunohistochemie. In de tumoren lijkt het belangrijke regulerende eiwit p53 onaangetast zodat vermoedelijk verschillende checkpoints functioneren. Bij veel paragangliomen zijn cel-populaties met een afwijkende DNA-inhoud aanwezig waarbij opvallend vaak sprake lijkt te zijn van verdubbeling of nagenoeg verdubbeling van het DNA (polyploidie). Dit duidt op een arrest in een laat checkpoint van de cel cyclus. Echter, in een TUNEL-assay bleek apoptose in de tumoren vrijwel afwezig te zijn, vermoedelijk door de aanwezigheid van het beschermende Bcl-x$_L$ eiwit.

Aan de hand van deze bevindingen is een hypothetisch model opgesteld dat tumorvorming in hoofd-hals-paragangliomen kan verklaren. Vermoedelijk is er sprake van een functionerend checkpoint dat leidt tot een laat arrest in de cel cyclus

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waarbij de DNA-replicatie nagenoeg compleet is. De gearresteerde cellen zijn ongevoelig voor apoptose dankzij expressie van een beschermend eiwit en stapelen zich op. Vermoedelijk weten enkele cellen aan het arrest te ontsnappen om vervolgens opnieuw de cel cyclus te doorlopen. Dit resulteert in een kleine fractie prolifererende cellen met een geringe potentie voor kwaadaardige (maligne) degeneratie.

Een intrigerend aspect van paragangliomen is de sterke histologische gelijkenis van de tumoren met het oorspronkelijke paraganglionweefsel. In veel gevallen vertonen de tumoren dezelfde architectuur en organisatie in celnesten (Zellballen) opgebouwd uit chief cellen met omgevende sustentacular cellen. Hoewel er consensus bestaat dat paragangliomen voornamelijk bestaan uit een neoplastische proliferatie van chief cellen blijft de aard en rol van de sustentacular cellen onduidelijk en controversieel. Sommige wetenschappers beschouwen paragangliomen als bifasische tumoren met twee verschillende typen tumorcellen terwijl anderen menen dat alleen de chief cellen neoplastisch zijn en dat sustentacular cellen afkomstig zijn uit het omgevende stroma. De aard van de sustentacular cellen is nader onderzocht in **Hoofdstuk 5**. Sustentacular cellen kunnen goed worden geïdentificeerd met de marker S-100. Door suspensies van tumorcellen te labelen met deze marker konden sustentacular cellen van de chief cellen worden gescheiden en gesorteerd in een flow-cytometer. De sustentacular cellen bleken een normale DNA-inhoud te bezitten zonder verlies van wild-type SDHD allel terwijl chief cellen afwijkende DNA inhoud bezaten en het wild-type SDHD-allel hadden verloren. Uit deze resultaten kan worden geconcludeerd dat de sustentacular cellen niet tot de tumorpopulatie behoren en waarschijnlijk een stromale oorsprong hebben. Derhalve zijn de chief cellen de enige neoplastische component in paragangliomen.

Naast hoofd-hals-paragangliomen omvat het tumorspectrum ook feochromocytomen. Deze tumoren zijn vaak hormonaal actief en hebben een grotere neiging tot maligne gedrag. Naar analogie van mutaties in het SDHD-gen bij hoofd hals paragangliomen zijn in feochromocytomen ook mutaties in SDH-genen aangetroffen waarbij tot heden voornamelijk sprake bleek te zijn van afwijkingen in het SDHB-gen. Tot heden was echter onduidelijk in hoeverre SDHD-mutaties ook aanleiding geven tot ontwikkeling van feochromocytomen. In samenwerking met de afdeling Endocrinologie is daarom een klinische studie verricht naar het

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voorkomen van feochromocytomen bij patiënten met SDHD-gerelateerde hoofd-hals-paragangliomen. De resultaten van deze studie worden besproken in **Hoofd-stuk 6**. Bij 8 van 40 opeenvolgende patiënten met een SDHD-gerelateerd hoofd-hals-paraganglioom was sprake van een synchroon of metachroon feochromocytoom. Bij vijf verwijderde feochromocytomen van deze patiënten bleek in de tumorcellen het wild type SDHD-allel verloren te zijn gegaan zodat alleen het gemuteerde SDHD-allel resteerde wat vermoedelijk aanleiding heeft gegeven tot de tumorontwikkeling. Geconcludeerd wordt dat ontwikkeling van feochromocytomen bij patiënten met SDHD- hoofd-hals-paragangliomen relatief frequent voorkomt en dat aanvullend onderzoek naar aanwezigheid van feochromocytomen bij deze patiënten geïndiceerd is.

De resultaten van dit proefschrift hebben geleid tot verder inzicht in verschillende facetten van de tumorbiologie van hoofd-hals-paragangliomen. Zo zijn de effecten van SDH-genmutaties in de tumorcellen nader onderzocht (genotype-fenotype relaties). Een mogelijke hypoxie-gerelateerde signaalroute via paracriene of autocriene effecten van bFGF in paragangliomen is nader bestudeerd. De klinisch indolente groei van de tumoren is gecorreleerd aan een zwakke proliferatieve activiteit. Dit wordt mogelijk veroorzaakt door een combinatie van een zwakke mitogene stimulus met een functioneel checkpoint-arrest in de cel cyclus en een gebrek aan apoptose. Dit samen resulteert in een klein aantal prolifererende cellen met neiging tot polyploidisatie en geringe maligne progressie. Aanvullende studies dienen te worden verricht om dit hypothetische model te toetsen en te verdiepen en zullen in de toekomst hopelijk leiden tot parameters die het klinisch gedrag van deze tumoren kunnen voorspellen. Daarnaast is de aard van sustentacular cellen en het mogelijk bifasische karakter van paragangliomen nader onderzocht. Uit genotypering van dmv flow-cytometrie gesorteerde cel-populaties blijken de sustentacular cellen niet tot de tumorcellen te behoren maar vermoedelijk van stromale oorsprong te zijn. Daarom moeten hoofd-hals-paragangliomen worden beschouwd als een monoklonale proliferatie van chief cellen. Als laatste is gebleken dat er sprake is van een aanzienlijke prevalentie (20%) van feochromocytomen bij patiënten die bekend zijn met SDHD-gerela-teerd hoofd-hals-paragangliomen. Screenen op feochromocytomen bij deze groep patiënten lijkt dan ook geïndiceerd.

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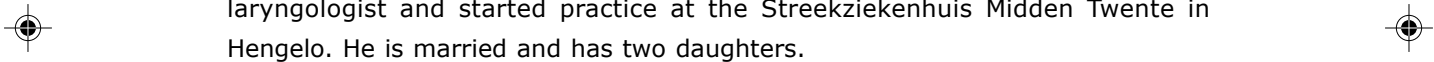
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Curriculum Vitae

Pieter Bas Douwes Dekker was born on the 8th of August 1966 in Delft, The Netherlands. He attended secondary school (VWO) at the Rijnlands Lyceum in Oegstgeest and graduated in 1984. After graduation he participated in an exchange program as an exchange student for one year in East Brunswick, NJ, USA and graduated from East Brunswick High School in 1985. He started his study of Biomedical Sciences at Leiden University in 1985 and graduated in 1991. After his military service he commenced Medical School at the Rijksuniversiteit Leiden in 1993 and obtained medical qualification in 1997. He worked as an AGNIO-resident at the Bronovo Hospital in The Hague for one year. In 1998 he was attached to the department of Pathology of the Leiden University Medical Center where he conducted his research on Head and Neck Paragangliomas under supervision of Prof. C.J. Cornelisse and Dr. A.G.L. van der Mey. In 2000 he commenced his clinical traineeship as a resident at the department of Otorhinolaryngology of the Leiden University Medical Center under supervision of Prof. J.J. Grote, Prof. R.J. Baatenburg de Jong and Prof. J.H.M. Frijns. In 2005 he obtained his registration as an otorhino-laryngologist and started practice at the Streekziekenhuis Midden Twente in Hengelo. He is married and has two daughters.
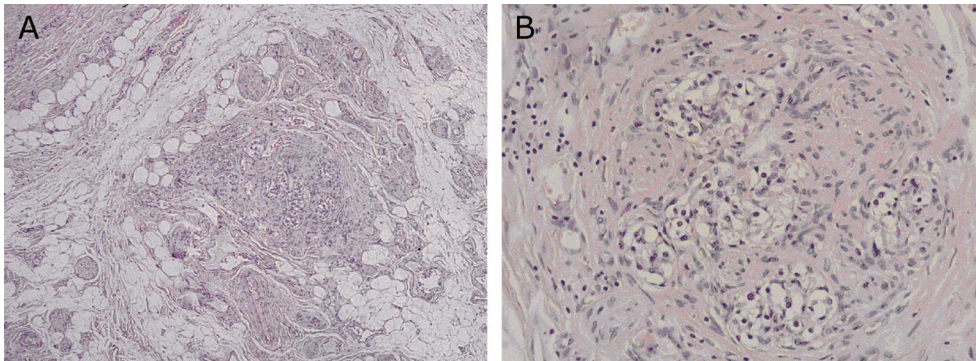
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Figure 1.4: A) Section of a carotid body paraganglion situated in the adventitial plane of the carotid bifurcation (HE, 20x), **B)** Section of a carotid body paraganglion demonstrating the '*Zellballen'* architecture with clusters of chief cells surrounded by sustentacular cells at the periphery, situated in a highly vascularised stroma with unmyelinated nerve endings (HE 100x).
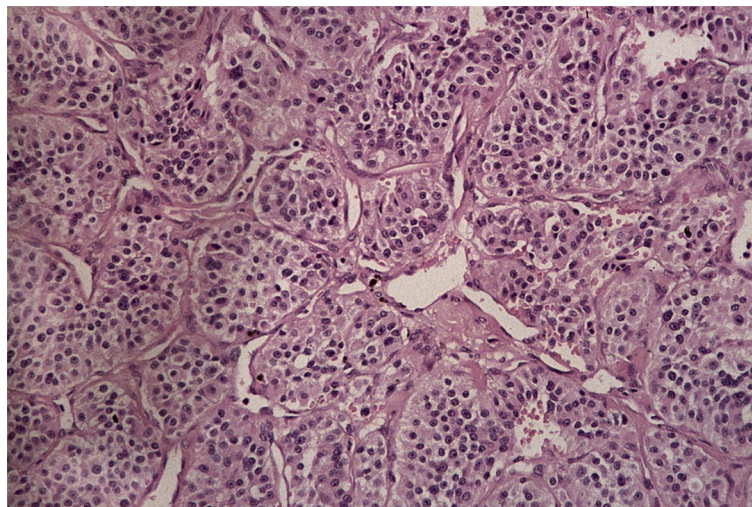

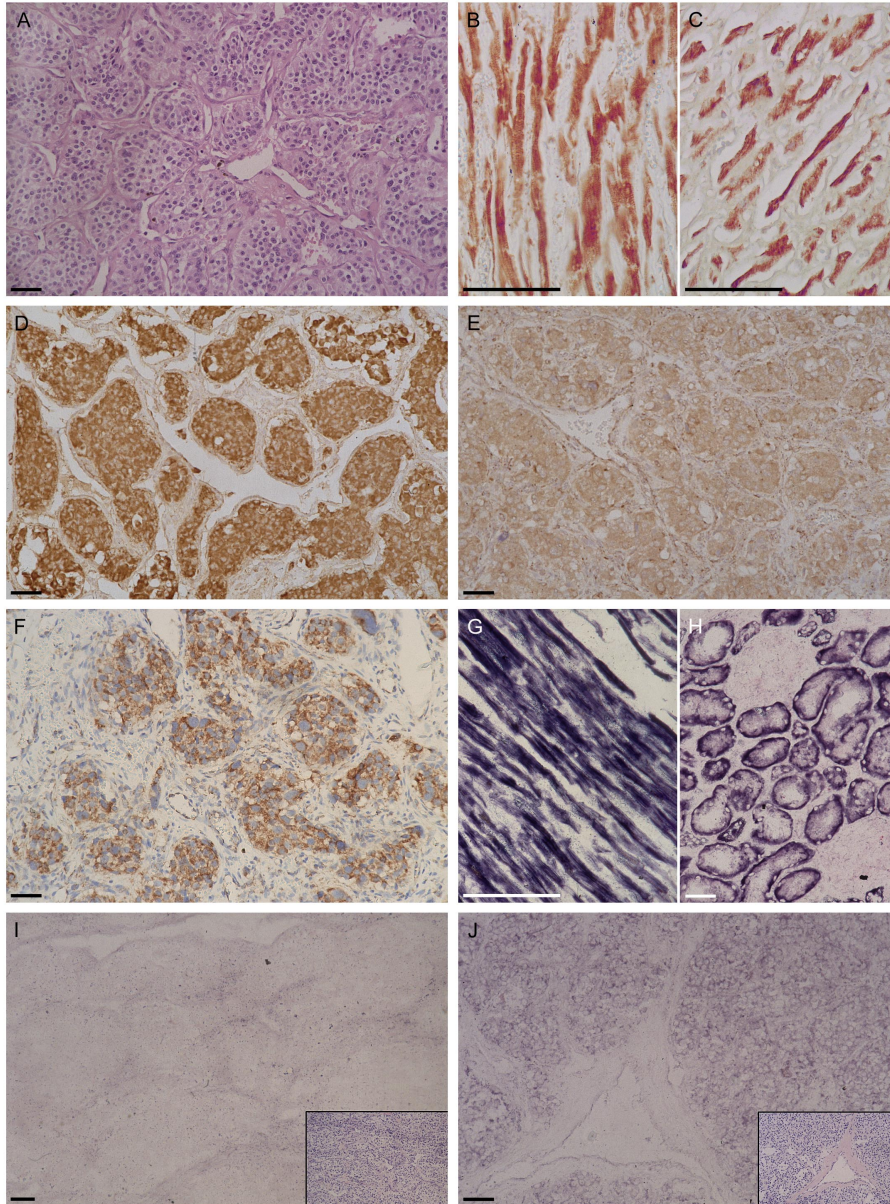**Figure 1.7:** Head and Neck Paraganglioma, typical '*Zellballen'* architecture (HE 25X).

Figure 2.1: Routine, immunohistochemical and enzyme-histochemical images of head and neck paragangliomas and control tissue. Inserted scale bars represent 0.05 mm. A: paraganglioma: Zellballen-pattern, B: mAb-Fp staining in myocardium; positive control, C: mAb-Ip staining in myocardium; positive control, D: strong cytoplasmic staining with mAb-Fp in paraganglioma (PGL1), E: weak cytoplasmic staining with mAb-Ip in paraganglioma (PGL1), F: strong cytoplasmic staining with mAb-Ip in paraganglioma (sporadic tumour 20), G & H: SDH-activity in myocardium and kidney tubular epithelium, I: (absent) SDH-activity in chief cells of a paraganglioma (PGL1), insert: HE-staining, J: (positive) SDH-activity in chief cells of a paraganglioma (sporadic tumour # 20) 25x, insert: HE-staining
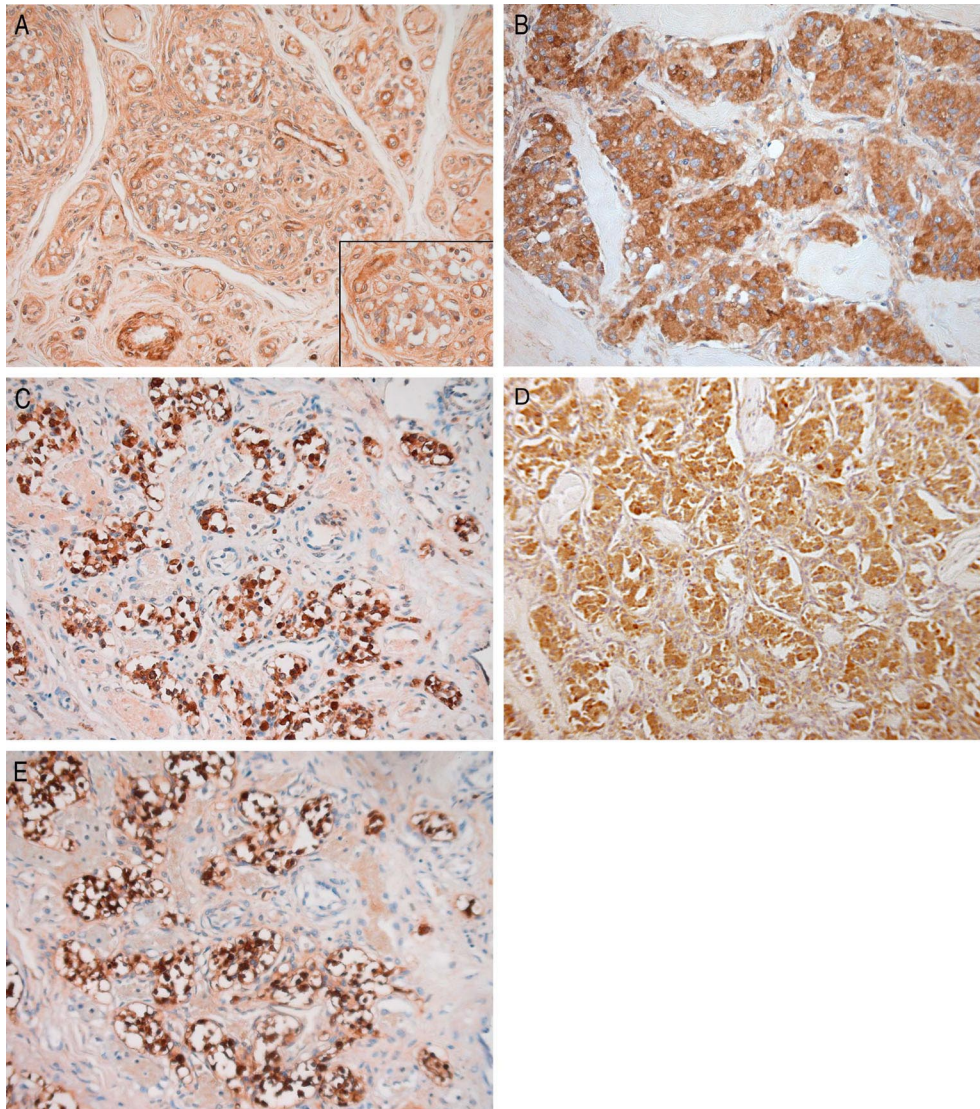
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Figure 3.1: Immunohistochemical images of carotid body-paraganglia and head and neck paragangliomas. A) FGFR1-sraining of carotid body, 150x, *insert*: chief cells within Zellball, ~300x. B) FGFR1-staining in paraganglioma, 100x. C) bFGF-staining of carotid body, 150x. D) bFGF-staining in paraganglioma, 100x. E) Chromogranin-A-staining in carotid body, 150x.
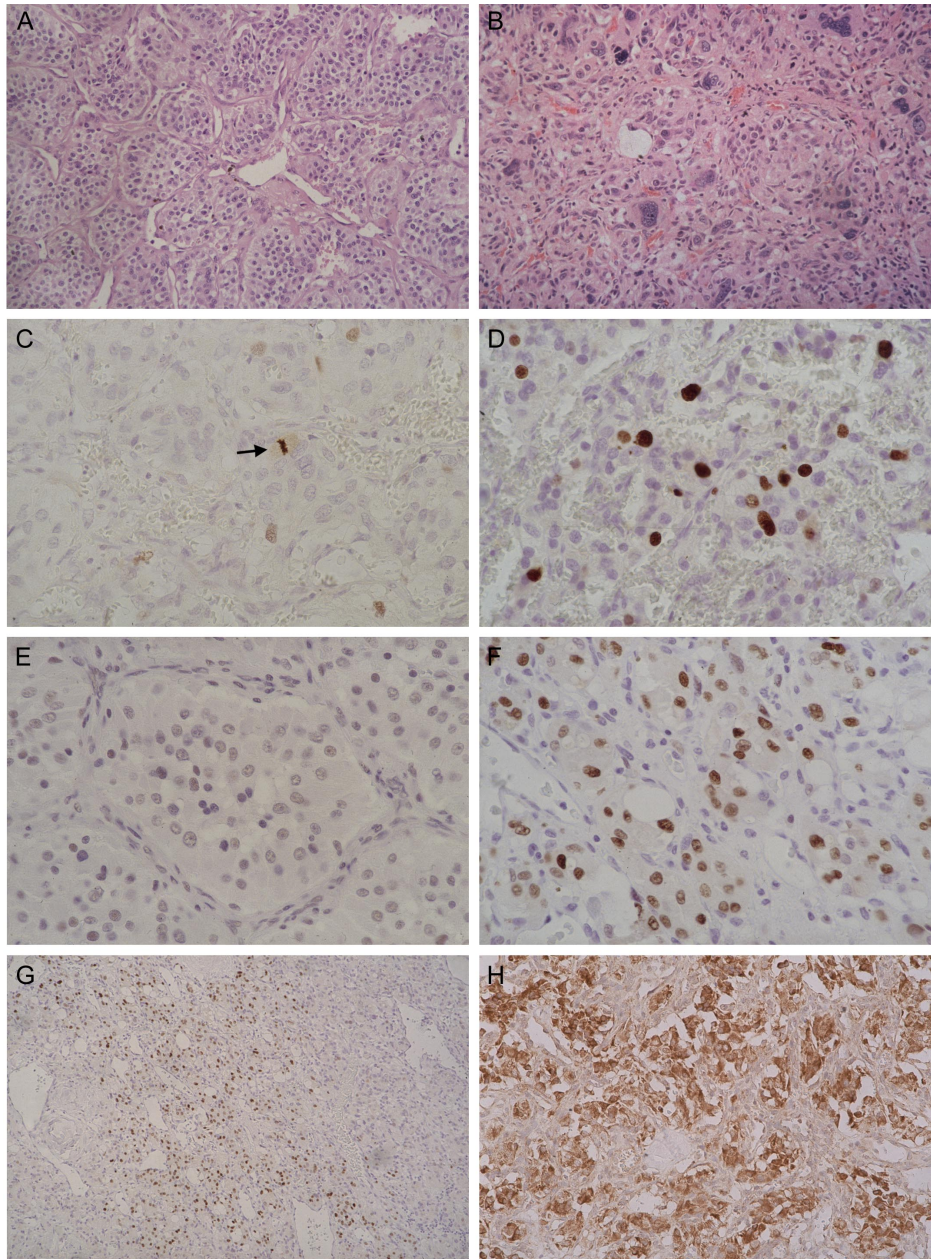
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Figure 4.3: Histology and immunohistochemistry of head and neck paragangliomas. A: HE-staining 25x, B: Nuclear pleomorphism and giant nuclei, HE-staining 50x, C: mitotic figure (black arrow), Ki-67 staining 100x, D: scattered nuclear staining of Ki-67, 100x, E: faint nuclear staining of Tp53, 100x, F: nuclear staining of p21^{waf}, 100x, G: clustering of p21^{waf} staining, 12.5x, H: strong granular cytoplasmic staining of Bcl-x$_l$, 50x.
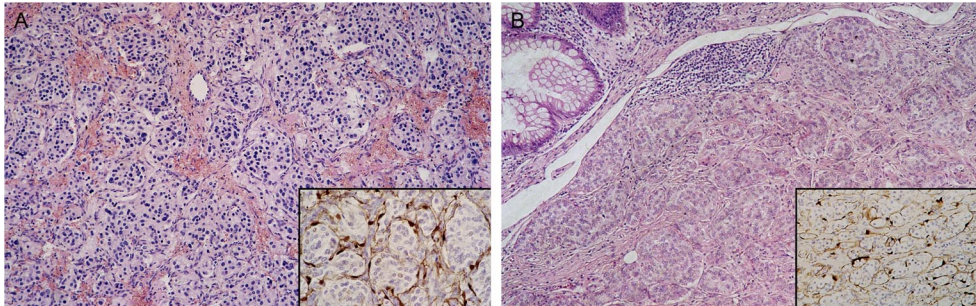
Figure 5.1: *A)* Light-micrograph displaying characteristic Zellballen pattern in paraganglioma, HE 25x, *insert*: immunohistochemical staining showing positive sustentacular cells in paraganglioma, S-100; 100x *B)* Light-micrograph of appendicular carcinoid, HE; 25x, *insert*: immunohistochemical staining showing positive spindle cells in carcinoid, S-100; 25x.

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