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ARTICLE

Morphological-Histochemical Study of Intestinal Carcinoids and K-ras Mutation Analysis in Appendiceal Carcinoids

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Intestinal carcinoids are potentially malignant neoplasms. Their histogenesis and pathogenesis are currently uncertain. The morphological and histochemical characteristics of twenty intestinal carcinoids are studied. The primary sites of three mucin-producing tumors were examined by electron microscope. Furthermore 11 appendiceal carcinoids were analysed by the polymerase chain reaction (PCR) for the detection of ras and p53 point mutations. Microscopically all carcinoids were of mixed type. Focal mucin production was evident in three carcinoids that metastasised to regional lymph nodes. HID-Alcian blue staining proved that mucin in both primary and secondary foci did not belong to the sulphated group. The secretory granules and mucin droplets found in a single neoplastic cell suggest that carcinoids of the small intestine and some of the appendix arise from the endoderm. Neither ras nor p53 mutations were detected. It seems that ras oncogenes are probably not involved in the pathogenesis of appendiceal carcinoids. (Pathology Oncology Research Vol 5, No 3, 205–210, 1999)

Keywords: carcinoids: morphology, histogenesis, p53 mutations, ras oncogenes

Introduction

In 1938 Friedrich Feyrter described a system of cells dispersed in the gut and other parts of the body. He proposed that these cells constitute a diffuse endocrine organ. In 1968, Pearse proposed the concept of a widely dispersed system of endocrine cells presumed to be of neural crest origin in order to explain the histochemical and ultrastructural similarities of a variety of endocrine cells and tumors derived from them; ie. neuroendocrinomas or APUDomas that are characterized by Amine Precursor Uptake and Decarboxylation. The acronym APUD is commonly used to refer to these cells.

The histogenesis of neuroendocrine tumors is obscure. While it is well known that certain APUDomas originate from the neural crest, this has not been proved for intestinal carcinoids, the origin of which has given rise to debate and controversy.[2,22] The fact that the same neural markers are found in both APUD and other cells militates against the theory that APUD cells originate from the ectoderm. Molecular studies in transgenic mice have shown that the existence of a common genetic region, which regulates the transcription of some protein amongst neural, APUD and other cells, does not constitute evidence of a common origin of the tissues.[2]

Since the pathogenesis of carcinoids is still unclear, the role of oncogenes and tumor supressor genes in their development has to be determined. Among these two important groups of transforming genes, the ras family of proto-oncogenes and the p53 tumor suppressor gene are the most frequently altered in sporadic colorectal neoplasms.[14,17] Actually, it has been suggested that ras gene mutations are involved in tumor progression through cooperation with p53 inactivation. The oncogenes of the ras family (N-ras, H-ras and K-ras at chromosomes 1, 11 and 12 respectively) are activated by point mutations at codons 12, 13 or 61.[8] They encode membrane bounded 21kD proteins with GTPase activity, involved in cellular signal transduction. Mutation at the specified codons leads to a state of continuous signal transduction, causing the constitutive stimulation of proliferation. The clinical importance

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of the p53 tumor-suppressor gene, as one of the most frequently mutated genes in a variety of human neoplasms, has been appreciated in recent years.[17] Damage of DNA results in p53-mediated arrest in the G1 phase of the cell cycle, in order to allow DNA repair. If the degree of damage is too great for effective DNA repair, p53 can induce apoptosis. Almost all point mutations occur within conserved domains II–V of the p53 gene and alter the function of the p53 protein in a dramatic way.

The molecular study of carcinoids should be considered as complementary to the clinical and histologic examination since cancer is thought to be a genetic disease that develops following the accumulation of multiple genetic alterations. Although the accumulation of point mutations -including K-ras and p53 genes- in the process of sporadic colorectal carcinogenesis has already been reported their involvement in the oncogenesis of carcinoids has not yet been determined. Moreover, the existence of different genetic pathways that do not involve K-ras or p53 mutation has to be considered.

In this study histopathologic, histochemical and ultrastructural analysis of 20 intestinal carcinoids was conducted in an attempt to investigate their histogenesis. Furthermore mutational analysis of ras and p53 genes was performed in an effort to determine the possible relationship between the presence of mutation and the patients clinicopathologic characteristics.

Materials and Methods

Tissue specimens

Our material was collected from archives covering a decade at the Pathology Department of the University of Athens. Twenty cases (11 males and 9 females) aged 7–85 (mean age 36.3 years) comprising single, mid- and hindgut carcinoids, were studied. Thirteen neoplasms were located in the appendix, 4 in the ileum and 3 in the rectum. Specimens were fixed in 10% buffered formalin and staining of paraffin sections (4 μm) with hematoxylin-eosin, HID-Alcian blue and PAS-Alcian blue was performed. The diagnosis and morphological classification of the tumors into five types (A, B, C, D and mixed) were made on the basis of their histologic pattern (insular, trabecular, tubular, neoplasms of lower differentiation and combinations of all these) respectively.[32] In each case, ten optical fields were examined and the number and shape of the cellular aggregates were determined and percentages calculated.

Histochemistry

Special stains were used to detect mucin production by neoplastic cells and to investigate its composition. Sulfomucin was detected by high iron diamine (HID)-Alcian blue at pH 2.5. In order to detect the mixture of neutral and acidic mucins (sialomucin) PAS-Alcian blue stain

Table 1. **Histological and histochemical findings***

Case Nr	*Histologic pattern*					*Mucin secretion*	*PAS-Alcian blue*	*HID-Alcian blue*
	A	*B*	*C*	*D*	*Mixed*			
1**	85%	–	15%	–	A+C	–	–	–
2	95%	–	5%	–	A+C	–	–	–
3	80%	–	20%	–	A+C	–	–	–
4	–	4%	96%	–	B+C	–	–	–
5	80%	20%	–	–	A+B	–	–	–
6	90%	8%	2%	–	A+B+C	–	–	–
7	33%	67%	–	–	A+B	–	–	–
8	20%	80%	–	–	A+B	–	–	–
9	70%	10%	20%	–	A+C	–	–	–
10	68%	25%	7%	–	A+B+C	+++	+++ (blue)	–
11	70%	–	30%	–	A+C	–	–	–
12	90%	10%	–	–	A+B	–	–	–
13	–	20%	80%	–	B+C	–	–	–
14	12%	68%	20%	–	A+B+C	+++	–	–
15	80%	–	20%	–	A+C	–	+++ (magenta)	–
16	5%	70%	25%	–	A+B+C	+++	–	–
17	90%	–	10%	–	A+C	–	–	–
18	–	4%	–	96%	B+D	–	+++ (magenta)	–
19	70%	28%	2%	–	A+B+C	–	–	–
20	–	10%	–	90%	B+D	–	–	–

*All cases were negative with HID-Alcian blue
**1–13 appendiceal, 14–17 ileal, 18–20 rectal carcinoids

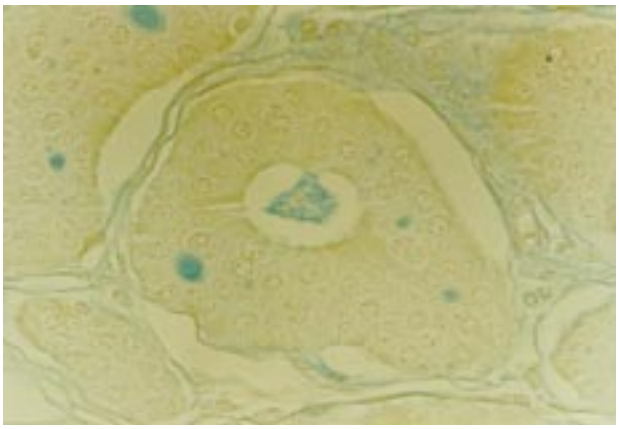

Figure 1. *Ileal carcinoid. Intracytoplasmic and extracytoplasmic localization of sialomucin (negative HID-Alcian blue x300)*

was used. HID-Alcian blue was applied in accordance with Spicer's criteria.[33] Normal colonic mucosa was used as a positive control for both stains.

Electron microscope

Tissue obtained from paraffin blocks was deparaffinized, and minced into small pieces. The fragments were postfixed in 2% buffered osmium tetroxide and embedded in Epon. Ultrathin sections, post-stained with uranyl acetate and lead citrate were examined by transmission electron microscope (Philips 300).

Molecular analysis

Genomic DNA extracted from paraffin-embedded tissue was amplified in a Thermal Cycler using the following thermal profile: denaturation at 94°C for 1 min, primer annealing at 55°C for 1 min, primer extension at 72°C for 1 min for 40 cycles. One primers used was modified in order to create cleavage sites for restriction enzymes. In brief, PCR for K-ras generates a 157 bp fragment that is cleaved twice by BstNI if K-ras gene is wild type, creating bands of 114 bp, 29 bp and 14 bp. The presence of mutation at codon 12 is indicated by a band of 143 bp. Hph I was used for detection of K-ras codon 13 aspartic acid mutation. PCR of H-ras first exon creates a fragment of 312 bp enclosing two Msp I cleavage sites (CCGG).

Specific primers were used to amplify a selected region of p53 gene encompassing the hot-spot codons 248-249 and 273-278. Restriction enzyme analysis of the PCR products was performed using the appropriate enzymes (Mspl for codon 248, Haelll for codon 249, BstNI for codon 273 and BstUI for codon 278).

PCR products were also screened for mutations at the conserved domains IV and V of p53 gene by single-strand conformation polymorphism analysis (SSCP). Electrophoresis conditions were optimised for each reaction, respectively. After an initial step of heat denaturation the samples were electrophorised in a 1x MDE gel (FMC, USA). In general, constant power (3–9 Watt) was used for 16–17 hours.

Results

Histology

Microscope evaluation and classification of the tumors into five histological types were made using paraffin sections stained with haematoxylin and eosin. *Table 1* shows that all neoplasms were of mixed type, composed of cellular configurations varying in shape. Insular and tubular shapes (A+C) represented the highest percentage (35%) followed by A+B+C (25%), A+B (20%), B+C (10%) and B+D (10%).

Histochemical findings

Focal mucin production was detected in 3 carcinoids (1 appendix, 2 ileum) which had given rise to metastases to regional lymph nodes. The mucin was produced mainly by tubules. The PAS-Alcian Blue stain (pH 2.5) showed that in the ileal tumors the mucin was composed of acid and neutral mucopolysaccharides. The reaction of appendiceal carcinoid mucin was acidic. The HID-Alcian Blue negativity showed that the acid mucopolysaccharides did not contain sulphate radicals *(Figure 1)*. The crypts of the normal mucosa from the right colon (controls) stained black *(Figure 2)*. The mucin-producing carcinoids and their metastases comprised 3 cellular types (A+B+C). Mucin proved to be mainly of neutral composition since it had stained red with PAS-Alcian Blue.

Ultrastructural findings

Electron microscope photographs showed that the neoplastic cells from all three primary tumors contained secretory granules and large droplets of mucin *(Figure 3)*. The granules were composed of a homogeneous high electron density substance, enveloped in a membrane. Granular size and shape varied from cell to cell, most being round. Oval, ellipsoid, concave granules were also seen.

Molecular analysis

The molecular study of K-ras gene mutations was carried out in 13 cases (11 appendix, 1 ileum and 1 rectum), as material from the remaining cases was not available. Two of them failed to provide sufficient DNA for the PCR reaction. The remaining cases (9 appendix, 1 ileum, 1 rectum) were studied by the polymerase chain reaction

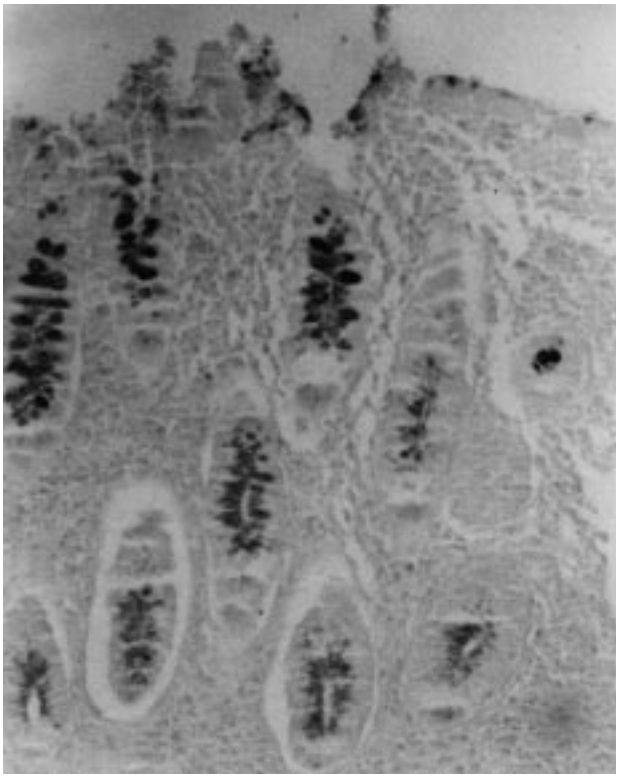

Figure 2. *Sulfomucins of normal colon stained positively (darkly) with HID-Alcian Blue (x200)*

and restriction-fragment length polymorphisms (PCR-RFLPs) in order to detect point mutations at codons 12 and 13 of K-ras, codon 12 of H-ras oncogene and hot spot codons 248,249 and 273,278 of p53 tumor suppressor gene.

No H-ras codon 12 or K-ras codon 12 or 13 -aspartic acid- mutations were detected in any of our specimens *(Figure 4)*. The hot spot codons 248, 249 and 273, 278 of p53 gene were not altered. Further p53 mutation analysis of domains IV and V did not reveal any mutation.

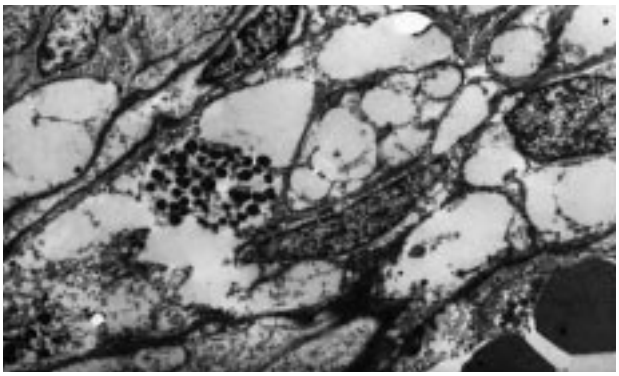

Figure 3. *Appendiceal carcinoid. Neoplastic cell containing mucin droplets and round secretory granules. Tissue obtained from paraffin blocks (x7000)*

Discussion

Apart from the classical histologic type of carcinoids[32] other forms (mucin-producing, composite tumors, goblet and clear cells) have also been described.[1,4,32] There is no agreement amongst scientists as regards the histological picture of carcinoids.[1,4,10,39] Our results were more compatible with those of other authors who found that 58.8% of ileal, appendiceal and rectal carcinoids were of mixed histologic type. We think that our morphological results are more realistic than those of other researchers[1,23,32] who support the single type of certain tumors. This is due to the fact that we did not focus only on the pattern which constitutes the major part of the neoplasm ignoring smaller areas having a different histologic picture.

The frequency of mucin-producing carcinoids varies in the diverse segments of the embryonic intestine.[20,23,32] Of our cases, 17.6% were mucin producing. To our knowledge mucin composition is not described precisely by other authors since for its investigation they used PAS-Alcian blue and Southgate's mucicarmine[9,20,32] which are not as specific as HID-Alcian blue. Applying the latter stain we showed that the acid mucin belonged to the carboxylated mucopolysaccharides. There was also a positive correlation between our results and those of other investigators, who reported a decrease or disappearance of sulphate mucopolysaccharides (HID-Alcian blue negativity) in the mucosa adjacent or covering malignant tumors or in colonic cancers with abundant mucin production.[15,28]

Electron microscopic examination proved that both mucin and neurosecretory granules are present in various normal tissues and numerous neoplasms.[12] There is controversy as to whether secretory granules and mucin are located in a single cell in goblet cell carcinoids.[1,26] To our knowledge no previous reports have elaborated on the ultrastructural features of the mucin producing variant of carcinoids. In our material we observed secretory granules and vacuoles with mucin existed in the same cell.

The histogenesis of intestinal carcinoids has been the object of much debate and questioning. Most authors believe that carcinoids of the appendix derive from the ectoderm, especially from subepithelial complexes found in the lamina propria of the GI tract mucosa.[5,6,18,24,29,30] Others support the endodermic origin theory[12] as they found an intense intraepithelial hyperplasia of the APUD cells in Lieberkuhn's crypts but no changes in the neurogenic complexes of the mucosa. The study of the histogenesis of jejunoileal carcinoids has produced conflicting results.[5,18,19,31] Moyana and Satkunam support the theory for the endodermal origin of small intestine carcinoids, but a large report disputes the results of the above authors.[31] Finally about carcinoids of the rectum it is claimed that they originate from modified subepithelial complexes.

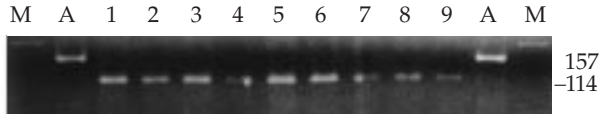

Figure 4. *K-ras codon 12 mutation detection in carcinoids by PCR amplification – BstNI digestion. The presence of a 114 bp band indicates absence of K-ras mutation at codon 12. A = undigested PCR product M = mw marker (øx 174/Hae III).*

Baylin in 1990 expressed two theories in order to explain the endodermic origin of pulmonary oat cell carcinoma and the link between APUD and other bronchial cells which give rise to non-small cell lung carcinomas. According to his second theory, during bronchial mucosa regeneration or, mainly, in pre-neoplastic states, all cells pass through an APUD differentiation. Baylin's views on pulmonary oat cell carcinoma histogenesis could perhaps by extension be applied to support the same embryonic origin of intestinal carcinoids. The description of composite tumors,[19] the detection of epithelial molecular indices (CEA, EMA, cytokeratins, etc) and the coexistence of secretory granules and mucin droplets in mucin-producing carcinoids in our cases constitute strong evidence of their endodermic origin.

In adenomas and adenocarcinomas of the colon, K-ras mutations at codons 12 and 13 are detected at high rates ranging from 25 to 50%[8,14], thus it appears that they play a significant role in the formation of these tumors. As far as small bowel adenocarcinomas are concerned, the percentage of K-ras mutations is lower, estimated to be around 14%.[36] On the other hand no K-ras mutations are detected in carcinoids of the small intestine,[36] while there is no mention of ras oncogene mutations in carcinoids of the appendix in the literature. Mutations of K-ras are also quite common in pulmonary adenocarcinoma (30%)[25] but not in lung neuroendocrine tumors of various differentiation.[25,34] Our results showed absence of such mutations in carcinoids of the appendix, indicating that they constitute an uncommon step in the pathogenesis of appendiceal carcinoids.

Mutation of p53 tumor suppressor gene is considered as one of the most common genetic abnormalities in human tumorigenesis. It is observed in 50% of different types of non-endocrine tumors. On the contrary, a small number of studies analysing abnormalities of p53 in carcinoids suggest that it may not be of equal importance to the pathogenesis of neuroendocrine tumors as it is not overexpressed in their great majority.[16,17,21,35] In carcinoids of the intestine, similarly to pulmonary ones, overexpression of p53 protein is absent.[17,21] Taking into consideration that negative immunostaining is not always an indicator for the absence of mutation in p53 gene,[16] we investigated the presence of mutations at hot spot codons of p53 gene by PCR-RFLP. The results were negative, supporting the idea that, despite its importance in the pathogenesis of gastrointestinal adenocarcinomas, p53 may not be involved in the development of neuroendocrine tumors of the intestine and especially of appendiceal carcinoids. Taking into consideration the results of our study as well as those of other researchers we conclude that different pathways exist probably for the development of neuroendocrine neoplasms and non-endocrine tumors arising at equivalent sites. Further studies are required to reveal the molecular genetic changes that lead to the formation of carcinoids

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