

## Replication Error-positive Samples Found in Pheochromocytomas

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**Abstract.** *Background: Adenomatous polyposis coli (APC) and E-cadherin (CDH1) tumor suppressor genes were investigated in human pheochromocytoma. Both genes are components of adherens junctions, but are also involved in wnt signalling in which one of the target molecules is c-myc protein. Materials and Methods: Fifteen sporadic pheochromocytomas were tested for gene instability by PCR/loss of heterozygosity. Detection of c-myc protein was performed using immunohistochemistry. Results: One sample with allelic imbalance of the APC gene and one with allelic imbalance of the CDH1 gene were found. Interestingly, another type of genomic instability was detected – replication error-positive samples (RER+). Four out of 13 heterozygous samples were RER-positive (30.8%). The instability is the result of impaired cellular mismatch repair. Immunohistochemistry showed increased levels of c-myc in comparison to normal adrenal tissue. Conclusion: Our results suggest that microsatellite genetic instabilities of the E-cadherin gene have a role in pheochromocytoma development and progression. Detected instability indicates that mismatch repair may be targeted in pheochromocytoma. Increased expression of c-myc protein as well as allelic imbalances of APC and CDH 1 genes suggest that the wnt signalling pathway may have a role in this malignancy.*

Pheochromocytomas are neuroendocrine tumors that produce catecholamines and originate from chromaffin cells, derived from the neural crest. They can cause endocrine hypertension by oversecretion of catecholamines. Pheochromocytomas are mostly located in the adrenal

medulla, but also in the ganglia of the sympathetic nervous system. These extra-adrenal pheochromocytomas occur in 15% of cases (1). About 10% of the pheochromocytoma cases are malignant. Forty percent of cases of the malignant tumor localize in the extra-adrenal tissues (1, 2). Pheochromocytomas may be either sporadic or a manifestation of a familial cancer syndrome, for instance Multiple Endocrine Neoplasia type 2 (MEN 2), von Hippel-Lindau (VHL) disease, or neurofibromatosis type 1 (NF 1).

Our knowledge of the etiology and pathogenesis of pheochromocytoma still needs to be elucidated, although great progress has been achieved along with the increasing advances in molecular genetics. Specific genetic alterations have been found to be associated with hereditary-predispositioned-pheochromocytomas (2-4). All patients with MEN 2 have germline mutations in the RET proto-oncogene (2, 5). Von Hippel-Lindau-associated pheochromocytomas exhibit mutations in the VHL tumor suppressor gene (6, 7) and patients with NF-associated pheochromocytoma show loss of the wild-type allele of the NF1 tumor suppressor gene (8, 9).

On the other hand, the genetic basis of sporadic pheochromocytoma is unclear, and much work is still required to determine the final list of genes involved, as well as the point in time of their activation/inactivation. Although it was suspected that the RET protooncogene may also be involved in the pathophysiology of sporadic pheochromocytoma, only about 8% of cases show RET protooncogene mutation. The mutations of the VHL and NF1 genes are also uncommon (10).

Our study analyzed genetic changes in sporadic pheochromocytoma with regards to the roles of two tumour suppressor genes—adenomatous polyposis coli (APC) and E-cadherin (CDH1). Both gene products are components of the adherens junction, where E-cadherin is bound to  $\beta$ -catenin, which in turn binds to the central part of the APC protein (11, 12). Our interest in elucidating the role of the APC gene stemmed principally from the findings that wild-type APC protein is highly expressed in the central nervous system and that there are strong

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indications that this wild-type APC protein is critically involved in the initiation of neuronal differentiation (13). In developmental processes the neural crest gives rise to sympathoadrenal progenitor cells. If a sympathoadrenal progenitor cell migrates into the adrenal primordial region, due to adrenal corticosteroids, it will differentiate into a chromaffin cell precursor and demonstrate endocrine chromaffin phenotype.

Besides the structural roles of the APC and E-cadherin genes in cellular architecture, the protein product of the APC gene plays a signalling role as a component of the wnt signal transduction pathway (14). APC acts as a negative regulator of the wnt signalling pathway, being a critical component of the beta-catenin destruction machinery heading to the proteasome. Wnt genes, together with other components of the wnt signalling pathway, are also implicated in cancer, especially in neoplasms of epithelial origin. Mutations in the adenomatous polyposis coli gene are responsible for familial adenomatous polyposis and the majority of sporadic colorectal cancers. However, a large variety of other cancers exhibit mutations of this suppressor gene (15). The APC gene (chromosome 5q21) (16) is organized in 16 translated exons and encodes a 2843 amino acid protein that is expressed in specific (frequently post-replicative) epithelial and mesenchymal cells of several fetal and adult human tissues.

The other gene investigated, E-cadherin (maps to 16q21), is one of the most important molecules of cell-cell adhesion in epithelial tissues. It is a member of a large family of genes that code for calcium-dependent cell-adhesion-molecules (17). The human epithelial E-cadherin gene encompasses 16 exons (18) and encodes a 120 kDa transmembrane glycoprotein that mediates cell-to-cell adhesion through homotypic interactions of its extracellular domain. A number of epithelial cancers demonstrate loss of expression as well as mutations of the E-cadherin gene. Recently, a tumour invasion/suppressor role has been assigned to this gene. Loss of heterozygosity on 16q is frequently detected in metastasizing malignancies derived from the liver, prostate and breast. Mutations in CDH1 have been described in a number of human cancers including breast, stomach, endometrium, ovary and thyroid (19). A transgenic mouse model with loss of E-cadherin expression developed invasive carcinoma from well-differentiated adenomas and germ-line mutations have recently been reported in early onset, diffuse-type stomach cancers (20). Nevertheless, classical cadherins—E- and N-cadherins being the best characterized—play important roles in the formation of tissues during gastrulation, neurulation and organogenesis (21). Another fact inspired us to analyze this gene in pheochromocytoma. There is a marked similarity between E-cadherin and ret-protein (22, 23). Although the extracellular domain of this transmembrane

tyrosine kinase has several similar repeats with putative  $\text{Ca}^{2+}$  binding motifs, the ret gene lacks matching of the splice sites to the CDH1 gene. It is possible that this kinase has acquired its cadherin-like motifs by convergent evolution (18).

One of the target molecules in the wnt signalling cascade is the c-myc oncogene product (24). Pheochromocytoma samples were also tested to the level of c-myc protein expression in order to determine potential changes.

The aim of our investigation was to detect genetic changes of two tumor suppressor genes, both components of the wnt pathway, in order to identify new molecular targets of neuroendocrine carcinogenesis.

## Materials and Methods

Fifteen sporadic pheochromocytomas, together with 15 autologous normal adrenal tissues, were collected from The Department of Pathology, Sisters of Charity University Hospital, Zagreb, Croatia. Tumor tissue was in all cases from the primary and not from a metastatic site. The samples were formalin-fixed and paraffin-embedded. Among those fifteen cases, 13 were benign and 2 malignant. All samples were located in the adrenal medulla and were unilateral. The age of patients varied from 28 to 66 years (mean age = 49.4), and there were 9 females and 6 males. The histopathological classification was pheochromocytoma.

The local Ethical Committee approved our study and all the patients gave their informed consent.

**DNA extraction.** Tumor samples used for DNA isolation were parts of the obvious tumor mass. Prior to DNA extraction, the pheochromocytoma samples were microdissected, so that the DNA extraction could be performed exclusively on tumor cells.

Twenty-micrometer paraffin sections were separated from the slide, transferred to a microtube and repeatedly extracted with xylene for a couple of hours. The tissue was then rinsed with absolute ethanol and incubated for two days at 37°C in digestion buffer (50 mM Tris-HCl, 1 mM EDTA, 0.5% Tween 20, pH 8.5) together with proteinase K. The digestion was stopped by heating the mixture for 10 min at 95°C (25).

One sample investigated was immediately frozen in liquid nitrogen and transported to the laboratory, together with 5 ml of corresponding blood sample in order to compare the amounts of DNA extracted from fresh tissue to the DNA from paraffin blocks and to optimize PCR reactions. Approximately 0.5 g of this tumor tissue was homogenized with 1 ml extraction buffer (10 mM Tris-HCl, pH 8.0; 0.1 M EDTA, pH 8.0; 0.5% sodium dodecyl sulfate) and incubated with proteinase K (final concentration 100 µg/ml; Sigma, USA) overnight at 37°C. Phenol chloroform extraction and ethanol precipitation followed. The corresponding blood sample was lysed with 7 ml distilled water and centrifuged (15 min/5000 g). The pellet was then processed as for DNA extraction from the tissue sample.

**Polymerase chain reaction.** The optimal reaction mixture (25 µl) for APC's exon 11 amplification was: 20 pmol of each primer (5'-GGACTACAGGCCATTGCAGAA-3' and 5'-GGCTACATCTCC AAAAGTCAA-3'), 200 µM of each dNTP, 200-400 ng template

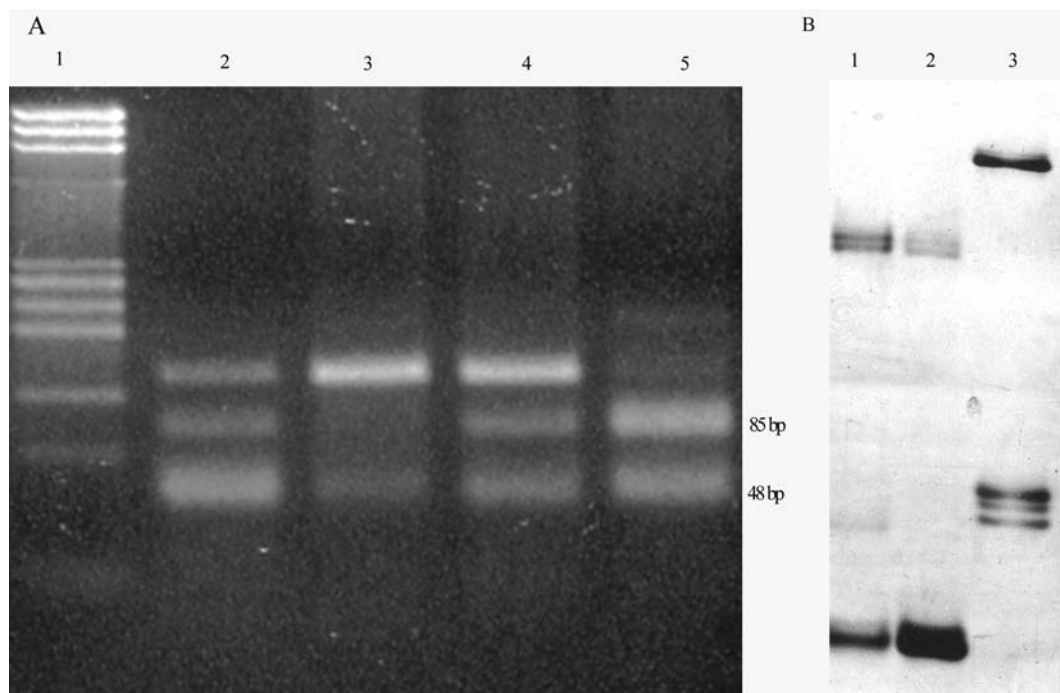


Figure 1. A) 3% agarose gel (stained with ethidium bromide) showing RFLP of the APC gene's exon 11. Lane 1 - molecular marker; lane 2- heterozygous normal tissue sample (patient no. 1); lane 3- homozygous normal tissue sample (patient no.11); lane 4- heterozygous normal tissue sample (patient no. 5), both alleles are shown; lane 5 - LOH of the APC gene in a corresponding pheochromocytoma sample. B) 13% polyacrylamide gel showing a pheochromocytoma sample suspicious of LOH. Lane 1 - DNA from normal tissue sample showing three bands; lane 2 - corresponding pheochromocytoma sample. Faint band at 133 bp demonstrates the allelic imbalance; lane 3 - molecular marker.

DNA, 1  $\mu$ l (0.5 U) of Taq polymerase, 1 mM  $MgCl_2$ , 5  $\mu$ l 10 X reaction buffer II (500 mM KCl, 100 mM Tris-HCl, pH 8.3). Polymerase chain reaction (PCR) conditions were: initial denaturation, 4 min/95°C; denaturation, 1 min/94°C; annealing, 2 min/58°C; extension, 1.5 min/72°C; 35 cycles.

The D16 S752 (GATA51G03) polymorphic region linked to the E-cadherin gene was amplified in a total volume of 25  $\mu$ l, each primer (5'-AATTGACGGTATATCTATCTGTCTG-3'; and 5'-GATTGGAGGAGGTGATTCT-3') 5 pmol, 200 ng DNA, 2.5  $\mu$ l 10X buffer II, 1.5 mM  $MgCl_2$ , 2.5 mM of each dNTP, 0.25 U Taq polymerase (Eppendorf, Germany). PCR conditions: initial denaturation, 3 min/96°C; denaturation, 30 sec/96°C; annealing, 35 sec/55°C; extension, 30+1 sec/72°C; final extension, 10 min/72°C; 35 cycles. The PCR products were analyzed on 2% agarose gels.

**Loss of heterozygosity.** Loss of heterozygosity (LOH) of the APC gene was detected on the basis of restriction fragment length polymorphism (RFLP) of the PCR products. A Rsa I polymorphic site in exon 11 was investigated. PCR amplification of exon 11 generated a 133- bp fragment that is cleaved to 85- and 48- bp fragments by Rsa I restriction if the polymorphic site is present, and remains uncut if the site is absent. PCR aliquots (10–15  $\mu$ l) were digested with 6 U Rsa I (Gibco, USA; 12 h at 37°C) and were electrophoresed on 3% agarose and 15% polyacrylamide gels. LOH/Rsa I was demonstrated only in informative (heterozygous) persons when the tumor DNA showed loss of either the single uncut

band (133 bp) or of the two cut bands (85+48 bp) compared to autologous blood DNA.

To discover loss of heterozygosity of the E-cadherin gene, a polymorphic marker D16S752 (GATA 51G03), linked to the CDH1 gene, was chosen from the Genome DataBase on the basis of its location, heterozygosity percentage and allele length. Heterozygous samples were visualized on 15% polyacrylamide gels run in 1X TBE (Tris/borate/EDTA, stained with silver) and on Spreadex EL 300 gels (Elchrom scientific, Switzerland), stained with SyberGold (Molecular Probes, Netherlands). Loss of heterozygosity of the CDH1 gene was established as one of the D16 S752 alleles missing in comparison to bands from the autologous blood sample.

**Immunohistochemistry.** Immunohistochemistry (IHC) was performed, according to the method described previously (26), using mouse monoclonal antibodies raised to human c-myc protein (diluted 1 : 100 in PBS/0.1% BSA) (Oncogene Science). Secondary antibodies were rabbit to mouse immunoglobulins (Dako) and the chromogen was DAB (Sigma).

The positive control for the IHC assay was normal adrenal gland medulla, while in the negative control primary antibody was omitted.

For each tumor the c-myc-positive cells were evaluated in at least four randomly selected areas. Each section was assessed independently by two observers. There was 95% initial agreement between the observers.

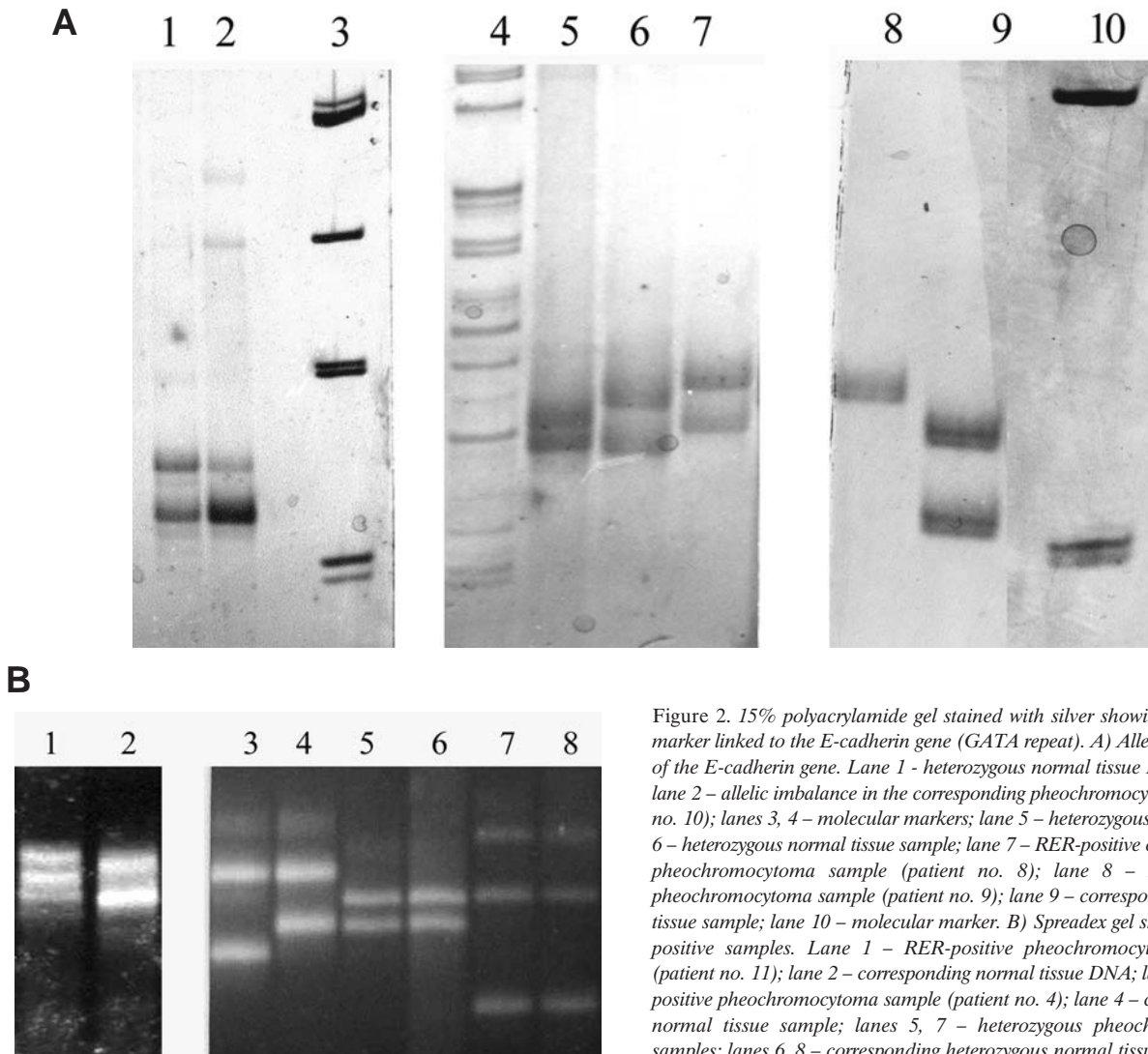


Figure 2. 15% polyacrylamide gel stained with silver showing D16 S752 marker linked to the E-cadherin gene (GATA repeat). A) Allelic imbalance of the E-cadherin gene. Lane 1 - heterozygous normal tissue DNA sample; lane 2 - allelic imbalance in the corresponding pheochromocytoma (patient no. 10); lanes 3, 4 - molecular markers; lane 5 - heterozygous sample; lane 6 - heterozygous normal tissue sample; lane 7 - RER-positive corresponding pheochromocytoma sample (patient no. 8); lane 8 - RER-positive pheochromocytoma sample (patient no. 9); lane 9 - corresponding normal tissue sample; lane 10 - molecular marker. B) Spreadex gel showing RER-positive samples. Lane 1 - RER-positive pheochromocytoma sample (patient no. 11); lane 2 - corresponding normal tissue DNA; lane 3 - RER-positive pheochromocytoma sample (patient no. 4); lane 4 - corresponding normal tissue sample; lanes 5, 7 - heterozygous pheochromocytoma samples; lanes 6, 8 - corresponding heterozygous normal tissue samples.

## Results

**Genomic instabilities.** First the Rsa I polymorphic site in APC's exon 11 was analyzed. From 15 pheochromocytoma samples analyzed, 11 (73.3%) were informative for this polymorphism. On 3% agarose gel one sample demonstrated LOH. We repeated the analysis of this sample on 13% polyacrylamide gel stained with silver and it showed a faint band at 133 bp. The sample was then reamplified and attacked with 10 U of Rsa I enzyme. The band reappeared on the polyacrylamide gel ruling out a problem of partial digestion. Bearing in mind that the tumor sample was microdissected, this finding led us to conclude that our sample comprised an allelic imbalance of the APC gene (1/11, 9%), as shown in Figure 1.

The polymorphic marker for the E-cadherin gene was highly informative 13/15 (86.6%), which was consistent with our previous results with this marker in renal carcinoma samples (27). We discovered three new allelic variants in the Croatian sample. Each allele differs by a 4-nucleotide repeat from the preceding one; therefore, allele number 1 is 102 bp long, while allele number 7 is 126 bp long (28).

From 13 informative patients, only 1 demonstrated allelic imbalance of the E-cadherin gene (7.7%), as shown in Figure 2.

When searching for LOHs of the E-cadherin gene, the D16 S752 microsatellite tetranucleotide marker revealed samples with replication error (RER)-positive phenotype. RER-positive samples have bands on different positions in comparison to bands of autologous normal adrenal tissue due

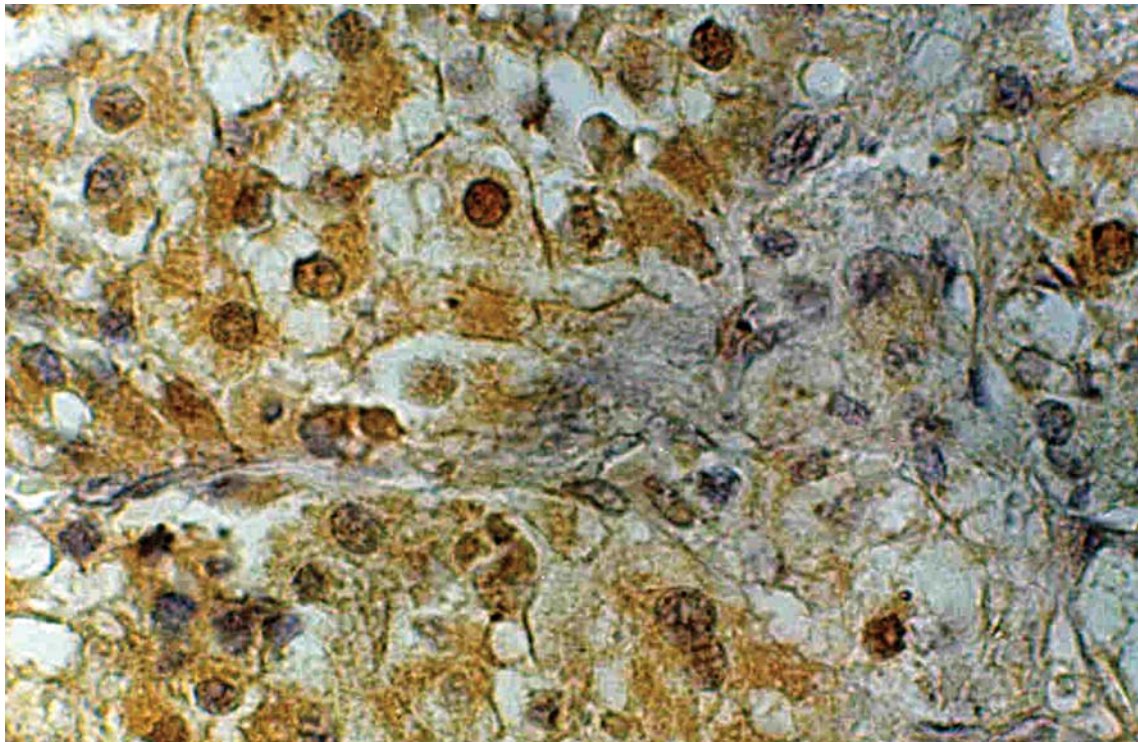


Figure 3. Immunohistochemistry showing c-myc protein in a malignant pheochromocytoma (patient no. 1). The protein is localized in nuclei or cytoplasm of cells.

Table I. Genetic changes of the APC and E-cadherin (CDH1) tumor suppressor genes and immunohistochemistry analysis (IHC) of c-myc product in pheochromocytomas.

Patient No.	APC gene exon11/Rsa I	E-cadherin D16S752	c-myc IHC
1*	heterozygous	heterozygous	+++
2	heterozygous	heterozygous	ND
3	heterozygous	heterozygous	ND
4*	homozygous b	RER+	+++
5	A. I.	heterozygous	+++
6	heterozygous	heterozygous	ND
7	heterozygous	homozygous	+
8	heterozygous	RER+	++
9	homozygous b	RER+	++
10	heterozygous	A.I.	+++
11	homozygous a	RER+	ND
12	homozygous a	heterozygous	++
13	heterozygous	homozygous	ND
14	heterozygous	heterozygous	+++
15	heterozygous	heterozygous	++

Heterozygous = without A.I. or RER+

a = both alleles 133 bp

b = both alleles have restriction sites (85+48 bp)

A.I. = allelic imbalance

RER+ = replication error-positive sample;

\*malignant

ND = not determined; adrenal gland IHC = +

to a defect in the replication/repair machinery in tumor cells (29). Interestingly, four pheochromocytoma samples were RER-positive samples (4/13, 30.8%) indicating, for the first time to our knowledge, that replication-repair machinery could be targeted in pheochromocytoma. All RER-positive samples were reamplified and repeatedly analyzed on both Spreadex and polyacrylamide gels (Figure 2).

Pheochromocytoma samples were also tested to the level of expression of the c-myc protein by IHC. Nine samples out of 10 available for IHC analysis (90%) showed increased levels of myc protein in comparison to normal adrenal tissue, as shown in Figure 3.

Genomic instabilities of two tumor suppressor genes investigated and the results of c-myc protein expression are summarized in Table I.

## Discussion

The mechanisms of neuroendocrine tumor initiation and progression have not yet been completely investigated and elucidated. As with other tumors, pheochromocytoma is the result of multiple consecutive genetic changes that represent a critical factor in tumor evolution. Although genes for mitochondrial complex II subunits (SDHB, SDHC and SDHD) have recently been suspected to be implicated in

sporadic pheochromocytoma development, new reports show that the frequency of their mutation is quite low and the findings on their role are inconsistent (30). Further susceptibility gene(s) remain to be identified.

The genes involved in tumor initiation and progression are not only those responsible for cell proliferation and survival, but also genes responsible for the control of cell adhesion and cell motility (17, 31). It is now apparent that tumor malignancy can, in certain aspects, be explained by alterations in the adhesive properties of neoplastic cells. With this in mind, we investigated two new candidates, APC and CDH1, tumor suppressor genes in a set of 15 sporadic pheochromocytomas.

Our analysis showed one sample demonstrating allelic imbalance of the APC gene, which indicates that gross deletions of APC are not the genetic basis of this tumor. Allelic imbalance of the CDH1 gene is also not very frequent, occurring in one sample, tested with a marker that is 86.6% informative. Allelic imbalances could not be the result of normal cells' DNA interference, because of microdissection. The explanation is that all tumor cells of the sample did not undergo this sort of alteration. The relatively low number of allelic losses of the APC and E-cadherin genes in our sample may be explained by random variation in pheochromocytoma, but also by the use of a limited number of markers.

Many immunohistochemical studies have examined changes in expression of the E-cadherin gene in human malignancies. In almost all non-colonic tumors examined, the patterns of changes in the expression of this gene are similar to those seen in colorectal cancer, *i.e.*, loss of protein expression is positively correlated with loss of tumor differentiation (17). Since the majority of the pheochromocytoma samples investigated in this study were benign and well-differentiated, as is typical with this tumor type, it is possible that E-cadherin alterations are destined to happen in later stages of pheochromocytoma progression. Finally, one must not rule out the additional genetic and epigenetic events that might have happened in our samples. E-cadherin promotor hypermethylation is known to be an inactivating event in some tumors (32) and APC gene hypermethylation has also been reported.

When searching for LOHs of the CDH1 gene, we came across a type of genomic instability that characterizes tumor development and progression—replication error RER. We found replication/repair machinery to be targeted in 30.8% of our sample. Considering that our analysis with only one marker revealed this frequency, our results suggest that, among other things, pheochromocytoma may be caused by a defect in the replication/repair machinery. Although the exact culprit for this defect in tumor cells is not known yet, deletion mutations may be due to slippage during replication/repair by strand

misalignment. Simple repeated sequences are genetically unstable, as judged by their increased mutation rate *in vivo* and *in vitro* and by their polymorphic nature in the human population (29, 33). The results obtained regarding RER+ samples are indicative of involvement of replication/repair genes (hMLH1 located on proximal chromosome 3p; or hMSH2, hPMS1, hPMS2) in pheochromocytoma, opening a potential new area of interest in neuroendocrine tumor studies. In both the sporadic and familial form of pheochromocytoma, allelic loss at 1p, 3p, 17p and 22q has been reported (34) and, according to the Weizmann Institute of Science Gene Card web site (<http://bioinfo.weizmann.ac.il/cards/>), chromosomes 1 and 3 harbor a lot of genes involved in human mismatch repair.

The genetic alterations found in our sample were not correlated with either age or sex. Genetically predisposed patients are younger at diagnosis of pheochromocytoma compared to patients with sporadic pheochromocytomas, which is consistent with the mean age of our patients (=49.4).

The increased levels of myc protein expression in pheochromocytoma as compared to normal adrenal tissue was also an interesting finding, and consistent with the results of other authors. Goto *et al.* (35) found the expression of c-fos and c-myc transcripts in pheochromocytoma to be constitutive. Since benign pheochromocytomas are characterized by cells with near absence of mitoses, our results might indicate the appearance of proliferative signals that precede malignant phenotype. The only sample that did not show increased c-myc expression in comparison to normal adrenal tissue (patient no. 7) harbored a point mutation in the ret oncogene (personal communication). This suggests that another carcinogenic pathway is targeted in this sample.

Evidence of mutation in different components of the single signaling pathway that have the same physiological consequences are very important for understanding carcinogenesis and hereditary diseases.

Our results suggest that microsatellite genetic instabilities of the E-cadherin gene have a role in pheochromocytoma development and progression. The detected instability indicates that mismatch repair may be targeted in pheochromocytoma. Increased expression of the c-myc protein as well as allelic imbalances of the APC and CDH1 genes suggest that the wnt signalling pathway may have a role in this tumor. This is, to our knowledge, the first indication of replication error involvement in the clinical evolution and progression of pheochromocytomas.

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