PHENOTYPE–GENOTYPE CORRELATION: CHALLENGE OF INTESTINAL-TYPE ADENOCARCINOMA OF THE NASAL CAVITY AND PARANASAL SINUSES

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Abstract: Background. Intestinal-type adenocarcinoma (ITAC) of the nasal cavity and paranasal sinuses shows microscopic features indistinguishable from colorectal cancer. Our aim was to verify whether the morphologic resemblances mirror genetic profile similarities.

Methods. Twenty consecutive surgically treated ITAC cases, previously investigated for p16INK4a and TP53, were investigated for hMLH1, hMSH2, and β-catenin immunoreactivity, and for adenomatous polyposis coli (APC), K-ras, and BRAF gene mutations.

Results. One case was immunonegative for both hMLH1 and hMSH2, and 12 tumors (40%) revealed a strong β-catenin over-expression. No BRAF and APC truncating mutations were identified, whereas K-ras mutations were detected in 9 ITACs (50%).

Conclusions. Our data confirm the phenotypic similarities at the genetic level between colorectal cancer and ITACs showing deregulation of K-ras/BRAF and loss of heterozygosity (LOH) of chromosome 18q. By contrast, both frequency rate and type of inactivation of the APC-β-catenin pathway differ in the 2 tumors, suggesting different gatekeeper events in the early development of ITAC (p16INK4a and TP53) and colorectal cancer (APC).

Keywords: ITAC; colorectal cancer; APC/β-catenin pathway, K-ras/BRAF pathway; mismatch repair genes

Intestinal-type adenocarcinoma (ITAC) of the nasal cavity and paranasal sinuses is an uncommon, aggressive professional-related high-grade tumor showing microscopic features that are usually indistinguishable from colorectal cancer.1,2 Morphologic resemblance between ITAC and colorectal cancer makes it reasonable to foresee that...
the 2 tumor types may share common or functionally equivalent genetic alterations.\textsuperscript{3–5}

Colorectal cancer is one of the most characterized tumors in terms of gene deregulation. Intensive screening for genetic alterations led to identification of 2 major subtypes of colorectal cancer, arising from distinct carcinogenic processes. The first, the microsatellite instability (MSI)-positive type, is characterized by normal karyotype, normal DNA index and genetic instability at microsatellite loci, due to the inactivation of DNA mismatch repair genes, in particular hMLH1 and hMSH2,\textsuperscript{6,7} as well as by activating mutations of \textit{BRAF} gene (up to 31\%).\textsuperscript{8} The second, designated the MSI-negative type, represents the most frequent sporadic colorectal cancer, occurring in about 90\% of cases, and is characterized by a combination of mutations in oncogenes or tumor suppressor genes in addition to epigenetic changes in DNA such as methylation of tumor suppressor genes.\textsuperscript{9,10} For this latter subtype of colorectal cancer, a model of carcinogenesis was proposed in 1990.\textsuperscript{9} This model foresees a colorectal cancer development where the temporal progression from healthy mucosa to carcinoma in situ is supported by mutations in adenomatous polyposis coli (\textit{APC}), \textit{K-ras}, \textit{TP53}, and deletion in colorectal cancer (\textit{DCC}) genes, occurring in about 80\%, 50\%, 55\%, and 55\% of cases, respectively,\textsuperscript{11} as well as by the inactivation of \textit{p16INK4a} through promoter hypermethylation in one half of the cases.\textsuperscript{9} In this subtype of colorectal cancer, \textit{BRAF} alterations rarely occur (up to 7\%).\textsuperscript{9}

By contrast, little is known about genetic alterations in ITAC. ITAC shows expression of CDX-2, cytokeratin 20,\textsuperscript{4,12} according to the colorectal cancer model; although at variance with colorectal cancer, ITAC shows no or only marginal occurrence of \textit{β}-catenin,\textsuperscript{13} \textit{K-ras}\textsuperscript{14–16} and \textit{TP53} mutations.\textsuperscript{14} Furthermore, the data regarding \textit{TP53} are controversial. In fact, through a more comprehensive methodologic approach, we observed a relatively high fraction of \textit{TP53} mutations (44\%) in ITAC, with a rate similar to that observed in colorectal cancer.\textsuperscript{3} Specifically, most \textit{TP53} mutations were G:C→A:T transitions and involved the CpG dinucleotides (50\%), as in colorectal cancer (54\%).\textsuperscript{17,18} In addition, we observed a high occurrence of loss of heterozygosity (LOH) at the 17p13 locus (58\%) and \textit{p16INK4a} promoter hypermethylation (67\%)\textsuperscript{3} gain with a frequency rate similar to colorectal cancer.\textsuperscript{19–21} Cumulatively, these findings strongly suggest the existence of similarities between ITAC and colorectal cancer also at the molecular level. However, whereas \textit{TP53} altera-

**MATERIALS AND METHODS**

**Patients and Specimens.** The study analyzed 20 consecutive and untreated cases diagnosed as ITAC of the nasal cavity and paranasal sinuses surgically resected at the Istituto Nazionale per lo Studio e la Cura dei Tumori of Milan from 1988 to 1998. The formalin-fixed paraffin-embedded tissues from all cases were previously investigated for \textit{TP53}, \textit{p14ARF}, \textit{p16INK4a}, and \textit{H-RAS} gene alterations.\textsuperscript{3} In this study, we used the same case numeration of our first previous study. Case 11 was excluded because of Bouin fixation. The clinical and morphologic features of patients and samples were previously described.\textsuperscript{3}

**Immunohistochemistry.** Immunohistochemical analysis was performed on 5-μm sections obtained from the most representative paraffin-embedded tissue block selected on the basis of hematoxylin-eosin—stained sections, as already described.\textsuperscript{11} Immunoperoxidase phenotyping was performed in all cases, using antibodies against the following markers: hMLH1 (clone G16815, Pharmingen International San Diego, CA; 1:50 diluted); hMSH2 (clone FE11, Oncogene Research, Boston, MA; 1:50 diluted), and \textit{β}-catenin (mouse monoclonal antibody Transduction Laboratories, Lexington, KY; 1:2000 diluted). All antibodies were diluted in a blocking solution containing 0.05 M phosphate-buffered saline (PBS), 1\%
bovine serum albumin (BSA), and 0.1% sodium azide. For hMLH1 and hMSH2 proteins, loss of expression in the tumor cells was considered when normal nuclear staining in adjacent non-neoplastic cells was observed. A sample of colorectal cancer carrying a germline hMLH1 mutation was used as the control. The β-catenin protein expression was evaluated for the presence of nuclear, cytoplasmic, and membranous accumulation in both tumoral and normal surrounding tissues. A sample of MSI-negative colorectal cancer carrying 2 distinct somatic APC mutations was used as the control.

Mutational Status of APC, K-ras, and BRAF. Microdissection from methylene blue-stained tissue sections and genomic DNA extraction were carried out as previously described. For each gene, amplified products were purified using the Qiamp Purification Kit (Qiagen, Chatsworth, CA) according to the manufacturer’s instructions and then directly sequenced on an ABI PRISM 3100 automated capillary Genetic Analyzer (Applied Biosystems, Foster City, CA) and analyzed with Sequencing Analysis and Sequence Navigator software programs. Each sequence reaction was performed at least twice, starting from 2 independent polymerase chain reaction (PCR) products.

APC. Codons 1286–1513 located in exon 15 and corresponding to the mutation cluster region (MCR) were analyzed by direct DNA sequencing according to a previous report.

K-ras. Mutational analysis of codons 12 and 13 of the K-ras oncogene exon 1 was performed using mutant-enriched PCR, according to a previous work on non-small cell lung cancer formalin-fixed paraffin embedded tissues and plasma samples. The method consists of 2 amplification steps (semi-nested PCR). During the first PCR step, an artificial restriction site (BstNI and BglI for codon 12 and 13, respectively) is introduced with the use of mismatched primers to distinguish between wild-type and mutant sequence. Wild-type amplicons were then digested by BstNI or BglI restriction enzyme, whereas mutant products were enriched for a second round of amplification. The PCR conditions and the primers used were previously described. The sequence of the primer 4B was: 5'-TCAAAGAATGGTCCTGCACC-3'. The final digestion products were electrophoresed on a 3% agarose gel, stained with ethidium bromide, and visualized by ultraviolet (UV) light. Mutant products were sequenced.

BRAF. We analyzed exon 15 of the BRAF gene through DNA amplification, using specific primers. The primers for BRAF analysis included the exonic sequence and at least 50 nucleotides of the flanking intronic sequences.

Loss of Heterozygosity Analysis. Nine sets of polymorphic microsatellite sequences (D18S478, D18S1102, D18S474, D18S64, D18S68, D18S61, D18S1161, D18S462, D18S70), spanning the entire long arm of chromosome 18, were obtained from Applied Biosystems. The 2 loci D18S474 and D18S64 flank the region where 3 tumor suppressor genes (DCC, SMAD4/DPC4, SMAD2) are located. PCR reactions, gel electrophoresis and the definition of LOH were performed as previously described.

Statistical Analysis. The chi-square test and Fisher’s exact test were used where appropriate in the comparison of the present results (ITAC) and those obtained in our previous work of sporadic colorectal cancer, which mirrors the data reported in the literature. All p values were 2-sided.

RESULTS

Immunohistochemical Evaluation of hMLH1, hMSH2, and β-Catenin. The analysis of both hMLH1 and hMSH2 mismatch repair gene expression was successfully performed in 19 of 20 ITACs, as case 12 was not evaluable for hMLH1. All but 1 case of ITAC showed nuclear immunoreactivity for the 2 proteins in both tumoral and normal tissues (Table 1). Case 20 resulted immunonegative for both the proteins in the tumoral tissue and positive in inbuilt control (normal mucosa).

The immunohistochemical evaluation of β-catenin expression was successfully performed in all cases. Twelve patients revealed an immunoreactivity limited to the plasma membrane, whereas the remaining cases (8 of 20 = 40%) showed a strong β-catenin overexpression in the plasma membrane as well as immunopositive staining in the cytoplasm and in the nucleus (Table 1).

Mutational Status of APC, K-ras, and BRAF in Intestinal-Type Adenocarcinoma. Genetic analysis of these molecular markers was performed in 18 cases, as no tumoral tissue was available for 2 patients (patients 5 and 10).

APC. Analysis of exon 15 of the APC gene was restricted to the MCR, corresponding to codons
1286–1513, where more than 60% of APC alterations are detected in colorectal cancer. We detected APC mutations in 5 of 18 (28%) ITACs (Table 1). In greater detail, 5 missense mutations were found in 4 ITACs, as case 8 showed 2 distinct alterations. In 2 cases (cases 8 and 19), the same missense mutation at codon 1420 was detected (CCC→CTC, Pro→Leu). Moreover, a silent alteration was detected at the third nucleotide of codon 1421 (case 12), resulting in a T→C mutation without amino acid change (AGT→AGC, Ser→Ser). All the APC mutations detected were transitions. Overall, no truncating mutations were identified, either in normal β-catenin expression cases or in those overexpressing such a protein.

K-ras. We screened for mutations at exon 1, where more than 95% of mutations are detected in colorectal cancer and in various neoplastic diseases, using mismatched primers specific for the 2 mostly affected codons: 12 and 13. We detected K-ras mutations in 9 of 18 (50%) ITACs (Table 1). Overall, 11

<table>
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<tr>
<th>Case</th>
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<th>hMSH2</th>
<th>β-Catenin</th>
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<th>K-ras* Exon 1</th>
<th>BRAF Exon 15</th>
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<td>12 GGT→GAT  (Gly→Asp)</td>
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</tr>
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</table>

17/18 = 94%  18/19 = 95%  8/20 = 40%  5/18 = 28%  9/18 = 50%  0/18 = 0%  8/10 = 80%

Abbreviations: APC, adenomatous polyposis coli; NE, not evaluable; NA, not analyzed; WT, wild-type; NL, not loss; L, loss; Gly, Glycine; Glu, glutamic acid; Pro, proline; Leu, leucine; Ser, serine; Lys, lysine; Asp, aspartic acid; Val, valine; LOH, loss of heterozygosity.

*Numbers represent the altered codon.
K-ras mutations were found, with 2 patients (patients 2 and 14) carrying a simultaneous alteration at codon 12 and 13 (Figure 1). All the alterations detected at codon 12 were transitions, and the most represented mutation was the change GGT → GAT leading to the amino acid change Gly → Asp (8 of 9 cases). At codon 13 level, 1 mutation was the classical GGC → GAC (Gly → Asp), whereas the other ITAC altered in this codon showed the very infrequent change GGC → GTC (Gly → Val), which is the only transversion mutation observed in the K-ras gene analysis. Overall, in no single case was the K-ras mutational pattern restricted to codon 13.

BRAF. Owing to the exiguity of material, the analysis was limited to exon 15, where more than 90% of BRAF mutations are detected (not only in colorectal cancer, but also in various neoplastic diseases) typically represented by the T → A change at nucleotide 1796. No ITAC showed BRAF alterations (Table 1).

Combined Analysis of Markers Investigated. Figure 2 represents the results of a combined analysis in 17 of 20 MSI-negative cases. The markers encompass those investigated here, eg, β-catenin overexpression and K-ras mutations, as well as those we reported in a previous work on the same series, eg, TP53 and p16INK4a.

Eight (46%) and 5 (30%) tumors showed 2 and 3 distinct alterations, respectively, whereas only 2 cases (12%) displayed a deregulation of all the genes. Only 1 case (6%) did not show any alteration. Overall, the most common mutated pathway was p16INK4a → TP53 (5 cases, 28%).

No significant associations were observed comparing the analyzed markers and the clinicopathologic parameters.

Loss of Heterozygosity Analysis in Intestinal-Type Adenocarcinomas. LOH analysis of 9 polymorphic microsatellite sequences located on the long arm of chromosome 18q was successfully performed in 10 specimens (Table 1). Five cases were not analyzed because of the absence of material (tumor in 2 and healthy mucosa in 3 cases), and 5 cases gave unevaluable amplifications. LOH was observed in 8 cases (8/10 = 80%) (Table 1).

DISCUSSION

ITACs morphologically recapitulate both usual types and subtypes of colorectal cancer.1,2 Recently,3 in a study of 21 consecutive surgically treated cases of ITAC, we found an occurrence of TP53 LOH and mutations along with p16INK4a promoter hypermethylation similar to that observed in colorectal cancer, leading to the tentative conclusion that these 2 different tumor types might share genetic resemblance. A genetic similarity was also corroborated by recent reports based on the expression of CDX-2, a nuclear transcription factor involved in intestinal organogenesis, in addition to cytokeratin-20, another marker proper of intestinal...
epithelia.\textsuperscript{4,5} To verify whether the morphologic resemblances mirror genetic profile similarities, we extended our analysis to genes involved mainly in colorectal cancer using all but 1 of the previously characterized ITACs as case material.

Considering the molecular markers involved in the MSI-positive colorectal cancer subgroup, we found a low occurrence of hMLH1 and hMSH2 null-immunophenotype (6\% and 5\%, respectively), in keeping with a recent report.\textsuperscript{13} Such frequency is not significantly different compared with that we previously observed in a series of sporadic colorectal cancer: ITAC, 1/19 = 5\%; colorectal cancer, 11/99 = 11\% (p = .597).\textsuperscript{11} It is noteworthy that, at variance with colorectal cancer, both hMLH1 and hMSH2 were deregulated in the unique negative case we observed (case 20). Thus, following the colorectal cancer findings, it appears that the DNA mismatch repair genes play a null or marginal role in the pathogenesis of ITAC, and therefore, the vast majority of ITACs belongs to the most frequent MSI-negative pathway, whose hallmarks consist of alterations of the APC-\(\beta\)-catenin pathway and mutations of the \(K-ras\) gene.\textsuperscript{9}

As for \(\beta\)-catenin, a critical mediator of the \textit{wnt} transcriptional response negatively regulated by \(APC\),\textsuperscript{26} we found, at variance with a recent investigation,\textsuperscript{13} a protein overexpression at plasma membrane as well as at cytoplasmic and/or nuclear level in 8 of 20 (40\%) ITACs, a finding consistent with the fact that the APC-\(\beta\)-catenin pathway plays a minor role in ITAC, in agreement with the conclusion drawn by a recent study.\textsuperscript{13} Thus, in terms of APC-\(\beta\)-catenin pathway, ITAC and colorectal cancer significantly differ (ITAC, 8/20 = 40\%; colorectal cancer, 88/88 = 100\% (p .001)).\textsuperscript{11} Moreover, we observed that the differences between the 2 tumors were not restricted to the incidence rate of the APC-\(\beta\)-catenin pathway alterations, but they also involved the deregulation mechanism of this pathway. In fact, in sporadic colorectal cancer the inactivation is mainly driven by inactivating mutations occurring in the MCR of \(APC\) exon 15 (up to 70\% in our previous work),\textsuperscript{11} whereas in ITAC no \(APC\) truncating mutations were detected in the same region. Such a finding is statistically significant: ITAC, 0/18 = 0\%; colorectal cancer, 54/77 = 70\%; (p .001). The 5 \(APC\) mutations detected in ITAC were exclusively missense mutations, which might not affect the APC protein activity. Unfortunately, owing to the exiguity of available material, we could not investigate the other mechanisms of inactivation of this pathway, such as \(APC\) promoter hypermethylation or LOH and \(APC\) exons 1–14 or \(\beta\)-\textit{catenin} mutations, which, nevertheless, play a minor role in sporadic colorectal cancer.

Regarding the \(K-ras/BRAF\) pathway, we started to investigate the \(K-ras\) oncogene mutation in codons 12 and 13, which occur in about one half of colorectal cancers.\textsuperscript{11} In keeping with colorectal cancer \(K-ras\) mutation rate, we found mutations in 9 of 18 (50\%) ITACs. Almost all the detected \(K-ras\) mutations were transitions like those found in colorectal cancer. Interestingly, this finding replicates that noticed about the type of \(TP53\) mutations in our previous investigation in ITAC.\textsuperscript{9} The only exception was represented by the mutation GGC→GTC at codon 13, which is not routinely found in colorectal cancer. Interestingly, \(K-ras\) mutations at codon 13 were restricted to 2 cases carrying a simultaneous \(K-ras\) mutation at codon 12, suggesting that codon 13 \(K-ras\) mutation could play a minor role in ITAC carcinogenesis. Our \(K-ras\) data are quite in contrast with previous investigations reporting a null\textsuperscript{14} or marginal\textsuperscript{15,16} occurrence of \(K-ras\) mutations (3\% to 8\%) in ITAC. These inconsistencies might be related to ethnic differences, different methodologic approaches, or merely to the few cases so far analyzed.

Furthermore, we focused our analysis on exon 15 of the \(BRAF\) gene, where the classical mutation V599E is located, which occurs in more than 90\% of \(BRAF\) altered neoplastic diseases.\textsuperscript{22} No \(BRAF\) mutations were found in our case material. The result was not unexpected considering that ITAC shares with colorectal cancer the MSI-negative pathway, where \(BRAF\) mutations are rare (7\%).\textsuperscript{8}

Finally, we found a high proportion (80\%) of LOH in the long arm of chromosome 18q, where 3 tumor suppressor genes (\(DCC\), \(SMAD2\), and \(SMAD4\)) relevant in colorectal carcinogenesis are located, suggesting that the loss of such a region is important also in ITAC development. In conclusion, the results of this genetic analysis confirm that ITAC and colorectal cancer share many similarities at the morphologic and molecular level. The vast majority of ITACs resemble colorectal cancer exhibiting MSI-negative pathway, and showing deregulation of \(K-ras/BRAF\) genes and LOH of chromosome 18q, involved in the early and late phases of colorectal cancer development, respectively. However, the 2 tumors differ from each other in terms of both frequency rate and type of inactivation of the APC-\(\beta\)-catenin pathway. Comparing the present findings with those we previously observed in the same series regarding
TP53 and p16INK4a, the high fraction of ITACs showing deregulation of both such tumor suppressor genes (88% and 58%, respectively) (Figure 2) in the absence of APC truncating mutation supports the notion that early development of ITAC and colorectal cancer may be driven by different gatekeeper events. Furthermore, our data, obtained by analysis of several molecular markers on the same case material, support and complement the hypothetical model of ITAC pathogenesis that was recently published, in which K-ras oncogene and chromosome 18q tumor suppressor genes may play an additional role in the maintenance of ITAC. Last, the recent demonstration that TP53 status represents a promising biomarker to predict response to chemotherapy in this tumor type further supports this assumption.

REFERENCES