CASE REPORTS

Multiple Hyperplastic Polyps in the Stomach: Evidence for Clonality and Neoplastic Potential

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The origin and neoplastic potential of gastric epithelial polyps remains an area of great interest, and treatment choices are a topic of controversy. This report describes a patient diagnosed with three concurrent hyperplastic gastric polyps that were studied for genetic alterations. The polyps were investigated for alterations in the *K-ras* oncogene and the *p53* tumor suppressor gene and for p21^{WAF1/Cip1} and MDM2 protein overexpression. In addition, loss of heterozygosity at several loci that are frequently involved in human cancer was analyzed, microsatellite instability, a hallmark of the "mutator" phenotype, was determined, and Epstein-Barr virus infection was investigated. All separate areas from the three independent polyps harbored the same activating point mutation in codon 12 of the *K-ras* oncogene, indicating a clonal origin. DNA sequence alterations in *p53* were not found, although high *p53* protein levels could be shown by immunohistochemistry in areas of carcinoma within the largest polyp. No alterations in any of the other molecular markers were observed. The results strongly favor a clonal origin of the three independent gastric polyps and support the notion that these hyperplastic polyps may carry a risk for malignancy.

Gastric epithelial polyps can be subdivided in adenomatous, hamartomatous, and hyperplastic polyps.^{1,2} Adenomatous polyps, which form a minority, are by definition neoplastic.³ Hamartomatous polyps are most often encountered in patients with Peutz-Jeghers syndrome, juvenile polyposis, or Cronkhite-Canada syndrome.⁴ The neoplastic potential of these hamartomatous polyps is not clear.⁵ Similarly, the nature and neoplastic risk of hyperplastic polyps is not known, and their proper management remains a topic of controversy. Especially when multiple hyperplastic polyps are present, they may harbor areas of carcinoma.⁶ It is believed that carcinogenesis in the stomach follows a multistep progression model

that is comparable with the adenoma-carcinoma sequence in the colorectum.⁷ In the intestinal type of stomach cancer, an accumulation of genetic mutations leading to oncogene activation and loss of tumor suppressor gene function may be associated with a gastric dysplasia-carcinoma sequence. However, the specific genetic changes accompanying this sequence are less well defined.⁸

Activation of *ras* oncogenes, a frequent finding in colorectal neoplasms, is rare in the stomach, although it was reported in a subset of patients at high risk for stomach cancer.^{9,10} Activation of the *K-ras* oncogene by codon 12 point mutations is a common and relatively early event in colorectal neoplasms. Alterations in the *p53* tumor suppressor gene, frequently associated with the transition of an in situ lesion into an invasive neoplasm in the colorectum,¹¹ most likely play a similar role in the stomach.^{10,12} Mechanisms to disrupt *p53* protein function include missense mutations in exons 5 through 8, often accompanied by allelic loss of the remaining functional *p53* allele.¹³ The normal (wild-type) *p53* protein, but not the altered *p53* protein, binds specifically to DNA and acts as a transcription factor for several genes.¹⁴ One of these genes, called *WAF1* or *Cip1*, encodes a 21-kilodalton protein (p21^{WAF1/Cip1}) that is a potent inhibitor for several cyclin-dependent kinases that control progression through the cell cycle.¹⁵ Expression of the p21^{WAF1/Cip1} protein can easily be detected by immunohistochemistry but does not always reflect *p53* status accurately.^{16,17} In addition to *p53* gene mutation, accompanied by loss of the wild-type *p53* allele, other mechanisms by which *p53* function can be compromised have been described. One of these mechanisms is inactivation through binding

Abbreviations used in this paper: EBV, Epstein-Barr virus; LI, labeling index; LOH, loss of heterozygosity; PCR, polymerase chain reaction.

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to the MDM2 protein.¹⁸ MDM2 is a nuclear protein whose overexpression leads to the inhibition of p53, a mechanism mostly found in sarcomas.¹⁹ Loss of p53 function may also occur through binding to viral proteins of which the SV40 Large-T protein is the prototype.²⁰ In the stomach, cytomegalovirus or the Epstein–Barr virus (EBV) may be present, and binding of one of the virally encoded proteins (cytomegalovirus immediate early antigen and EBNA-5) to p53 was reported previously.^{21–23}

Similar to observations regarding the colorectum, alterations in the adenomatous polyposis coli (*APC*) gene on the long arm of chromosome 5 and in the deleted colorectal cancer (*DCC*) gene on the long arm of chromosome 18 have been described in the stomach.⁸ The phenomenon of microsatellite instability has also been reported for the stomach as well as for the large bowel.²⁴

We describe a patient with multiple hyperplastic polyps harboring dysplasia and carcinoma in the stomach who was negative for *Helicobacter pylori*. The different areas of these polyps were analyzed for alterations using well-established molecular markers. Our study indicates that clonality rather than independent growth underlies this case of multiple gastric polyps, further supporting a dysplasia-carcinoma sequence in gastric carcinogenesis.

Materials and Methods

Patient Information and Samples

The patient was a 67-year old white woman with dyspepsia from whom three gastric polyps were removed by endoscopy. The polyps were located in the corpus of the stomach; they were macroscopically visible as separate lesions with normal-looking mucosa in between and had diameters of 6.5, 3.5, and 2.5 cm, respectively. The patient had no prior medical history and no family history of polyps or gastrointestinal cancer. After routine procedures of formalin fixing and paraffin embedding during which the polyps were completely enclosed, tissue blocks were cut into 4- μ m sections for immunohistochemistry, in situ hybridization, and H&E staining. At histopathologic examination, all three polyps were diagnosed as hyperplastic.² The largest polyp had a heterogeneous constitution, showed scattered foci of high-grade dysplasia,²⁵ and contained a small carcinoma that was confined to the mucosa (intramucosal cancer) (Figure 1). The stalk consisted of normal preexistent oxyntic mucosa. The smaller two polyps also showed areas of high-grade dysplasia. There was mild chronic nonspecific inflammation in the antral and fundic mucosa.

The *ras* mutational analysis and p53 immunohistochemistry were also performed in 10 additional sporadic hyperplastic stomach polyps (8 white women and 2 white men; age, 34–

Figure 1. H&E-stained sections from the three stomach polyps and immunohistochemistry for p53 and p21^{WAF1/Cip1}. (A–C) Overview of the large and smaller polyps, (D) nonneoplastic polyp mucosa, (E) focus of high-grade dysplasia, (F) area of intramucosal carcinoma, (G) diffuse p53 positivity in intramucosal carcinoma, and (H) p21^{WAF1/Cip1} positivity in high-grade dysplasia.

86 years; mean age, 69 years) from the archives; these polyps were negative for dysplasia or carcinoma.

Immunohistochemistry and Quantification

Immunohistochemistry for p53, p21^{WAF1/Cip1}, and MDM2 was performed as described previously.^{17,26} The following antibodies were used: DO-7 (DAKO, Glostrup, Denmark) to detect the p53 protein; Ab-1 (Oncogene Science, Cambridge, MA) to detect p21^{WAF1/Cip1}; and MDM2 (SMP14, Santa Cruz Biotechnology, Santa Cruz, CA) to detect the MDM2 protein. The presence of cytomegalovirus was investigated with an antibody (E13; Argene, Varilhes, France) against the immediate early antigen. For immunoquantification, counterstaining of the nuclei was performed with methyl green. This enables quantification of the immunostaining by an automated analysis system as described by us previously.²⁷ For this study, the PRODIT (professional digitizing interobserver tool) system was used. This system enables the calculation of the number

of positive cells as a percentage of the total number of cells that are hit in a random fashion within a depicted area and therefore avoids selection bias.²⁸

In Situ Hybridization for EBV

EBV infection was detected with a detection kit (DAKO) against EBER-1 and EBER-2 nuclear RNA molecules, followed by rabbit anti-fluorescein isothiocyanate and swine anti-rabbit conjugated with biotin as recommended by the manufacturer. Hybridization was visualized with avidin-biotinylated horseradish peroxidase and 3,3'-diaminobenzidine tetrahydrochloride as chromogen.

Detection of Point Mutations in *K-ras* Codon 12

For the analysis of codon 12 of the *K-ras* oncogene, the different areas of interest were accurately microdissected from the H&E-stained tissue sections, omitting the deparaffinization step. From the largest polyp, areas of hyperplastic, dysplastic, and cancerous mucosa in addition to the normal preexistent mucosa surrounding the stalk were separately collected. From the two smaller polyps, the dysplastic mucosa was also microdissected. Duplicate sections for each area were separately collected and used for DNA isolation. Areas around *K-ras* codon 12 were then amplified by the polymerase chain reaction (PCR) and tested with biotin-labeled allele-specific oligodeoxynucleotide hybridization to identify possible mutant sequences. Specific signals were detected with an enhanced chemiluminescence system (Amersham, Buckinghamshire, England). The DNA isolation, PCR, and hybridization conditions have been described previously.²⁹

p53 Sequence Analysis

Because the area with carcinoma in the largest polyp was positive for p53 immunohistochemistry, DNA from this area was used for mutational analysis for exons 5 through 8. Amplified PCR products were molecularly cloned in a plasmid vector, and DNA isolated from pooled clones was subjected to bidirectional sequence analysis using conditions described previously.^{17,30} Because no gene mutations were found using this approach, we extended our analysis to exons 2, 3, and 9 using the same protocol. (The oligodeoxynucleotide primers used for this analysis are made available by the authors on request.)

Analysis of Microsatellite Markers

Loss of heterozygosity (LOH) was analyzed using microsatellite markers for loci on chromosomes 5, 17, and 18.^{31,32} These markers were IL9 to detect LOH for *APC*, D17S513 to detect LOH for *p53*, and D18S38 to detect LOH for *DCC*. In addition, sequences from the markers D5S208 on 5p, D5S107 on 5q, D9S51 on 9q, and D12S60 on 12q were tested for possible microsatellite instability. PCR and analysis of the amplified products were performed as described previously.³³

Figure 2. (A) Analysis of *K-ras* codon 12 point mutations in polyp areas. Except for row 1, the directly amplified *K-ras* sequences were spotted on the left, and mutant-enriched PCR products were spotted on the right. WT, wild type. Row 1, positive controls for each filter; row 2, nonneoplastic area from the large polyp; row 3, dysplastic area from the large polyp; row 4, intramucosal carcinoma from the large polyp; row 5, dysplasia from the second polyp; row 6, dysplasia from the third polyp; row 7, normal stomach mucosa; row 8, unrelated sample with a known valine (Val) mutation; row 9, placenta DNA; and row 10, negative control (no DNA added to the analysis). All polyp specimens show the same aspartic acid (Asp) mutation in codon 12 of *K-ras*. (B) Analysis of marker D17S513 on chromosome 17, indicating possible loss of genetic sequences near the *p53* gene. Lane 1, negative control (no DNA added); lane 2, normal stomach mucosa; lane 3, nonneoplastic area from the large polyp; lane 4, dysplastic area from the large polyp; lane 5, dysplastic area from the second polyp; lane 6, dysplastic area from the third polyp; and lane 7, intramucosal carcinoma from the large polyp. No LOH at chromosome 17p was observed in any of the microdissected areas.

Results

When the different areas of the three polyps were analyzed for activating *K-ras* codon 12 point mutations, all showed hybridization to one mutant-specific probe, indicating the presence of an activated *K-ras* oncogene. This mutation was present in the different microdissected areas of hyperplasia, high-grade dysplasia, and carcinoma, whereas the normal mucosa was negative. The mutation involved a transition of a guanine into an adenine in position 2 of codon 12, resulting in the substitution of the normal glycine by aspartic acid in the protein (Figure 2A).

The p53 immunohistochemistry was negative in the normal and hyperplastic mucosa. The dysplastic areas

showed focal positivity (labeling index [LI], 0%), whereas in the intramucosal carcinoma, almost half of the nuclei (LI, 40%) were positive (Figure 1) and MDM2 staining was negative. Immunohistochemistry for p21^{WAF1/Cip1} was positive in the dysplastic (LI, 8%) and cancerous (LI, 18%) mucosa. Interestingly, the terminally differentiated foveolar cells of the preexistent oxyntic mucosa were also positive for p21^{WAF1/Cip1}.

Sequence analysis of exons 5 through 8 showed no alterations in the normal DNA sequence of the *p53* gene. Because inactivating mutations in other protein-encoding exons have been described, although with lower incidence, we extended our analysis to exons 2, 3, and 9 of the *p53* gene. Again, this additional analysis did not show any sequence alterations in the *p53* gene. We then analyzed LOH at 17p, where the *p53* gene is located and which frequently accompanies *p53* gene mutations, but no LOH was observed (Figure 2B). Analysis of the chromosomal areas of the *APC* gene (at 5q) and of the *DCC* gene (at 18q) did not show any alterations, although the marker at *DCC* was not informative. No shifts were observed with any of the used polymorphic microsatellite markers, precluding microsatellite instability at these sites, thus excluding a mutator phenotype in this patient. None of the analyses for the presence of cytomegalovirus or the EBV was positive.

In 1 of the 10 hyperplastic gastric polyps that served as controls, a mutation was found that also resulted in aspartic acid (GGT → GAT) substitution; the *p53* immunohistochemistry was negative, except for a few scattered positive nuclei occurring in the neck zone in 3 of the polyps.

Discussion

When multiple neoplastic lesions are present within the same organ, their possible clonal origin is often difficult to determine and may be impossible using conventional techniques. The use of newly developed molecular markers identifying well-established cancer-related genetic alterations in such cases may address this question with more accuracy. Our results in the present case of multiple gastric hyperplastic polyps indicates a clonal origin rather than an independent growth of the three removed stomach polyps. Although *ras* mutations comprise one of the most frequent oncogenic mutations observed in human malignancies, they are rare in neoplasms of the stomach. It is therefore unlikely that the same *K-ras* mutations in these three polyps occurred by chance. A possible explanation to account for this phenomenon is that the same and very specific carcinogenic hit resulted in a simultaneous outgrowth of the

polyps. Although this scenario has been reported in other organs, such as specific *p53* gene mutations associated with aflatoxin exposure in liver tumors,³⁴ there are presently no indications that this is the case in the stomach. Therefore, we favor the hypothesis that the progeny of a simple transformed cell has spread throughout the stomach and has established the different polyps at different sites of the stomach. The same clonal expansion underlying the presence of multiple tumors at the same time has been described in other organ systems, such as in the bladder and head and neck region.^{35,36} It is not difficult to envision how cells in the stomach can migrate to and attach at other sites. It is known that shedding of (normal) cells occurs in large quantities in the stomach.³⁷ The similarity of the molecular makeup of the three polyps in other regards lends support to this postulated clonal origin. Apparently, *ras* mutations can be encountered in hyperplastic polyps of the stomach without neoplastic change on rare occasions; the meaning of this finding is unclear. Similar observations in the colorectum, in aberrant crypt foci without dysplasia, seem to indicate that *ras* mutations per se are not sufficient to provide a dysplastic phenotype.³⁸

The trend in *p53* immunohistochemistry positivity with increasing degree of malignancy (negative in the hyperplastic mucosa, focally positive in high-grade dysplasia, and almost half positive in intramucosal carcinoma) was initially interpreted as an indication for the inactivation of the *p53* tumor suppressor gene because mutated *p53* can be shown by increased protein levels. However, we were unable to show the specific event leading to disruption of the *p53* gene: no *p53* gene mutations were found after extensive analysis of the full protein-encoding DNA sequence, no LOH was present at 17p, MDM2 protein expression could not be shown by immunohistochemistry, and no cytomegalovirus or EBV infection was found. The absence of LOH at 17p, in conjunction with the increasing p21^{WAF1/Cip1} protein expression that accompanied the *p53* positivity, is in fact a relative argument in favor of overexpression of a functional (wild-type) *p53* protein. The mechanism of such high *p53* protein levels remains puzzling, but it has been proposed that alterations outside the protein-encoding domains of *p53* may account for the Li–Fraumeni phenotype, which depends on inactivation of *p53*, in some families.³⁹ Truncating mutations in *APC* have been described as a determinant of dysplasia in the colorectum when it occurs in addition to *ras*-induced hyperplasia-aberrant crypt foci,³⁸ but LOH of 5q, the locus of *APC*, was not observed. Furthermore, a study of other polymorphic markers at different sites throughout the genome

did not show a mutator phenotype. From a practical point of view, the current case supports the idea that multiple hyperplastic polyps may carry a risk for malignancy.⁶ The observations are consistent with a dysplasia-carcinoma sequence in the stomach and therefore favor prophylactic surgical treatment of severe gastric dysplasia.⁴⁰ Finally, our data suggest that clonal expansion of transformed epithelial cells cause multifocal lesions in the stomach; the stomach should be therefore scrutinized when a single neoplastic lesion is encountered.

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