Absence of ras gene mutations in early gastric carcinomas

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Abstract

The aims of this study were to assess the prevalence and type of activating point mutations at codons 12, 13, and 61 of the Ki-, Ha-, and N-ras genes in a series of early gastric carcinomas in white patients and to correlate these ras gene mutations, if any, with the histological type (Lauren classification), the type of growth pattern, and with the *Helicobacter pylori* status. Haematoxylin and eosin and Giemsa stained sections from 45 formalin fixed, paraffin wax embedded early gastric carcinomas were used to assess the Lauren type, the type of growth pattern, and the antral *H pylori* status. DNA was extracted according to standard procedures. Mutations at codon 12 of the Ki-ras gene were examined with a polymerase chain reaction based restriction fragment length polymorphism (PCR-RFLP) method and dot blot hybridisation with allele-specific ³²P-labelled oligodeoxynucleotide (ASO) probes. All other ras genes were analysed with specific PCR amplification and dot blot hybridisation with ASO probes. Mutations were detected by overnight autoradiography at -70°C. Some 20 intestinal-type and 25 diffuse-type early gastric carcinomas were seen. According to growth pattern, there were 24 small mucosal type early gastric carcinomas, five superficial spreading type early gastric carcinomas, and 16 penetrating type early gastric carcinomas (four penetrating A type, 12 penetrating B type). *H pylori* was found in the antral mucosa of 28 early gastric carcinomas (62%). Activating ras gene mutations were not found. It was discovered that activating point mutations at codons 12, 13, and 61 of the Ki-, Ha-, and N-ras genes do not play a part in the development of early gastric carcinomas in white subjects, irrespective of Lauren type. Moreover, differences in biological behaviour between early carcinomas with different types of growth pattern are not related to these ras gene mutations. Finally, *H pylori* positive and *H pylori* negative gastric carcinomas cannot be discriminated on the basis of ras gene mutational analysis.

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The human ras proto-oncogene family includes the homologous Ha-, Ki-, and N-ras

genes, which code for closely related 21 kDa proteins (p21^{ras}). These proteins, located at the inner cell membrane, can bind GDP and GTP and possess intrinsic GTP-ase activity.¹ Activation of the ras genes can be due to gene amplification with enhanced expression of p21^{ras} proteins.² Activation is usually caused by point mutations at codons 12, 13, or 61 of the ras genes, however, which confer transforming properties to these proto-oncogenes. As these mutations are all localised to or near the GTP-binding domain, the intrinsic GTP-ase activity of the p21^{ras} proteins is nearly 100% reduced and the p21^{ras} proteins persist in their biologically active, GTP-bound state. This, in turn, leads to a continuous signal transduction, even in the absence of an external stimulus.³

Point mutations in the Ki-ras gene are uniformly reported to be common in human malignancies such as lung, pancreatic, and colonic carcinoma.⁴⁻⁶ In contrast, conflicting data on the frequency and type of ras gene mutations in human gastric carcinomas have been reported, varying from 0-35% of cases.⁷⁻¹⁴ Most of the previous studies on gastric carcinoma did not analyse all ras genes for the presence of point mutations and did not include early gastric carcinomas. Moreover, all but one of the previous studies did not discriminate between the intestinal and diffuse type carcinoma according to Lauren. As these two types not only differ in morphological, clinical, and epidemiological characteristics,¹⁵⁻¹⁷ but also differ in pathogenesis,^{18 19} it might well be that the genetic changes underlying both tumour types are also different. Interestingly, recent studies (both histological and serological) suggest that approximately 60% of all gastric carcinomas, irrespective of Lauren type, may be attributable to *Helicobacter pylori*.²⁰⁻²⁴ To date, however, no studies have been reported that have dealt with the issue as to whether or not *H pylori* positive and *H pylori* negative gastric carcinomas differ in molecular make up. Finally, although the prognosis of surgically treated early gastric carcinomas is generally excellent,²⁵ its metastatic potential and postoperative five year survival rate seem to be correlated with the type of growth pattern.²⁶ The mechanism(s) underlying these apparent differences in biological behaviour still remain(s) to be elucidated. Therefore, in view of the above, we have examined activating point mutations in the Ha-, Ki-, and N-ras genes with the following aims: (a) to assess the prevalence and type of ras gene mutations in a series of early gastric carcinomas in white patients and (b) to

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correlate the presence of these mutations, if any, with the Lauren type, the type of growth pattern, and the *H. pylori* status.

Methods

Tissues

Forty five gastrectomy specimens from patients with early gastric carcinomas, previously classified as either intestinal- or diffuse-type according to Lauren, were retrieved from the files. The tissues had been fixed in 10% buffered formalin and processed to paraffin wax by routine methods. Early gastric carcinoma was defined according to the Japanese Society of Gastrointestinal Endoscopy.²⁷ The tumour histology according to Lauren and the type of growth pattern according to Kodama (Fig 1) were assessed using the original paraffin wax embedded, haematoxylin and eosin stained sections. All tissue blocks of non-malignant antral mucosa were recut and stained by haematoxylin and eosin and Giemsa. Chronic atrophic gastritis, intestinal metaplasia, and *H. pylori* status were assessed using the criteria of the Sydney classification.^{28, 29} *H. pylori* was judged absent when both staining methods were negative for the micro-organism. All parameters were independently assessed by two of the authors (MC/PB). In rare cases of disagreement, consensus was reached after discussion.

DNA isolation

Six serial 5 µm sections were cut from each paraffin wax embedded tumour block. A haematoxylin and eosin stained section was prepared to permit precise localisation of the tumour area and to estimate the proportion of tumour cells present. Then, the tumour area was selectively scraped off, thereby increasing the number of tumour cells to at least 50% of the total cell population. After collection in separate Eppendorf reaction tubes (each containing five sections), 200 µl of a lysis buffer (10 mM TRIS HCl (pH 8.3), 1 mM EDTA, 0.2% Tween 20, and Proteinase K (20 µg/µl)) was added. After overnight incubation at

56°C, the samples were heated for 10 minutes at 100°C to inactivate Proteinase K. After 10 minutes of centrifugation at 12 000 rpm, the supernatant was removed and stored at 4°C for later use. Additional serial sectioning of each tumour block for other related studies showed that the tumours were still present and had not been cut out of the blocks.

PCR-RFLP analysis of Ki-12 ras gene mutations

To detect Ki-12 ras gene mutations, a polymerase chain reaction based restriction fragment length polymorphism (PCR-RFLP) analysis was used.³⁰⁻³³ Briefly, this technique uses a two stage PCR amplification. In the first reaction, a Mva I (BstNI) restriction enzyme recognition site is generated in the Ki-12 ras wild-type allele. After incubation with Mva I, the non-cleaved (mutant-enriched) DNA is again amplified with a different primer pair. The first PCR was performed in a reaction mixture containing 5 µl of 10× PCR buffer (35 mM MgCl₂, 150 mM (NH₄)₂SO₄, 600 mM TRIS HCl pH 8.4), 1 µl of primers A and B (50 pmol/µl), 2.5 µl of deoxynucleoside triphosphates mixture (2 mM each), 0.2 µl Taq polymerase (5 U/µl, Perkin-Elmer Cetus, Emmerlyville, USA), 5 µl of extracted template (slide) DNA and H₂O up to a final volume of 50 µl. Each mixture was overlaid with two drops of mineral oil. The PCR profile was as follows: five minutes at 96°C for one cycle, two minutes at 96°C, one minute at 55°C, one minute at 73°C for one cycle, followed by 23 cycles of 30 seconds at 96°C, 30 seconds at 55°C, one minute at 73°C. Thereafter, Mva I digestion was performed at 37°C for one hour in a final reaction volume of 25.5 µl, containing: 12.5 µl of the first PCR product, 2.5 µl of Mva I adjustment buffer, 0.5 µl of Mva I (10 U/µl, Boehringer), and 10 µl H₂O. The second PCR was performed under similar conditions as the first PCR with minor modifications; primer B was replaced by primer C, 1 µl of Mva I digested DNA served as template DNA, whereas the PCR profile consisted of five minutes at 96°C for one cycle, two minutes at 96°C, one minute at 55°C, one minute at 73°C for one cycle, followed by 35 cycles of 30 seconds at 96°C, 30 seconds at 55°C, one minute at 73°C.

All PCR-RFLP products were tested by dot blot hybridisation to verify the presence or absence of mutations.

PCR amplification of other ras-specific sequences

Amplification of Ki-13, 61, and all Ha and N-ras sequences (codons 12, 13, 61) was performed as described by Slebos *et al.*³⁴

Dot blot hybridisation with allele-specific oligodeoxynucleotide probes

For each oligodeoxynucleotide (ASO) probe, one Nitran N membrane (Schleicher and Schuell) was cut and attached to a glass plate. One microlitre of the PCR products was spotted onto the membranes. The membranes

Figure 1: Types of growth pattern of early gastric carcinomas according to Kodama *et al.* (adapted from ref 26); m=mucosa, sm=submucosa. Small mucosal type; early gastric carcinomas <4 cm with mucosal or only slight submucosal invasion. Superficially spreading type; early gastric carcinomas >4 cm confined to either mucosa or submucosa. Penetrating (Pen) A type; early gastric carcinomas <4 cm and growing expansively with complete destruction of the muscularis mucosae. Pen B type; early gastric carcinomas <4 cm and growing infiltratively with fenestration of the muscularis mucosae.

Oligonucleotides for Ki-12 ras gene mutation analysis

	Sequence
Primers*	
A (5' amplimer)	ACTGAATATAAACTTGTGGTAGTTGGGACCT
B (3' amplimer)	TCAAAGAATGGTCCTGGACC
C (3' amplimer)	TAATATGTCGACTAAAACAAGATTACCTC
Allele-specific oligonucleotides (ASO)†	
K-12 wild-type	GTTGGACCTGGTGGCGTAGG
K-12 a	GTTGGACCTTGTGGCGTAGG
K-12 b	GTTGGACCTAGTGGCGTAGG
K-12 c	GTTGGACCTCGTGGCGTAGG
K-12 d	GTTGGACCTGTTGGCGTAGG
K-12 e	GTTGGACCTGATGGCGTAGG
K-12 f	GTTGGACCTGCTGGCGTAGG

*Mismatched nucleotides, †mutant nucleotides.

were air dried and thereafter exposed to ultra-violet irradiation for two minutes for DNA cross linking. To analyse mutations of the Ki-ras gene at codon 12, allele-specific ³²P-labelled ASO probes were used as listed in the Table. For all other ras gene mutations, ASO probes were used as described by Verlaan-de Vries *et al.*³⁵ All probes were purchased from Pharmacia. Before labelling, the membranes were heated at 96.8°C in 0.1 standard saline citrate for 10 minutes. Labelling of each probe and subsequent hybridisation procedures were performed as described by Kern *et al.*³⁶ Mutations were detected by overnight autoradiography at -70°C.

Validation of the mutational assays

In a previous study by Slebos and Rodenhuis,³⁴ it was shown that the conventional mutational assays – similar to those used in our study – were able to detect a 5% subpopulation of cells containing a specific ras gene mutation among 95% of cells with the wild-type sequence (1:20). Analogous to their experiments, we validated our PCR-RFLP technique for the detection of Ki-12 ras gene mutations by mixing DNAs of two different adenocarcinoma cell lines: GLC-A1 (wild-type sequence) and NCI-H23 (codon 12 mutation). After amplification according to our PCR-RFLP protocol, dot blot hybridisation was performed as described. The PCR-RFLP technique detected a 0.4% subpopulation of mutant cells among 99.6% wild type cells (1:250) (data not shown).

Statistics

The χ^2 test was used for statistical analysis. A p value <0.05 was considered significant.

Results

Histology

There were 20 intestinal-type and 25 diffuse-type early gastric carcinomas. Chronic atrophic gastritis was present in 35 gastrectomy specimens. Intestinal metaplasia was found in the surrounding mucosa of 95% (19 of 20) intestinal-type early gastric carcinomas and of 52% (13 of 25) diffuse-type early gastric carcinomas (p<0.01). In the intestinal metaplasia positive cases, moderate/severe intestinal metaplasia was found in 68.4% (13 of 19) of

intestinal-type early gastric carcinomas and in 23.1% (three of 13) diffuse-type early gastric carcinomas (p<0.05). *H. pylori* was present in the antral mucosa of 14 intestinal-type (70%) and 14 diffuse-type early gastric carcinomas (56%) (NS).

Growth pattern

Twenty seven early carcinomas (60%) were confined to the mucosa and 18 (40%) extended into the submucosa. According to growth pattern, there were 24 small mucosal-type early gastric carcinomas (all limited to the mucosa), five superficially spreading-type early gastric carcinomas (three mucosal, two sub-mucosal), and 16 penetrating type early gastric carcinomas (four penetrating A type, 12 penetrating B type).

Mutational analysis

Positive and negative controls were included in all series of mutational assays and were run contemporaneously with the test cases to minimise the risk of false positive and false negative results. DNA from tumours previously shown to contain ras gene mutations served as positive controls. DNA from tumours known to contain only the wild-type sequence were used as negative controls. Distilled water was also used as a control in all assays. All tumours tested showed a positive hybridisation signal for the wild-type sequence. After high-stringency washing of the blots, however, activating point mutations of the ras genes were found absent in all early gastric carcinomas, irrespective of Lauren type, type of growth pattern, and *H. pylori* status (Fig 2).

Discussion

Our results show that activating point mutations at codons 12, 13, and 61 of the Ki-, Ha-, and N-ras genes apparently do not play a part

Figure 2: Dot blot hybridisation for Ki-12 ras gene mutation detection. Top: autoradiogram after non-stringent washing; bottom: same autoradiogram after high stringency washing: only the positive control shows a signal.

in the development of gastric carcinomas of white subjects. In contrast, studies on gastric carcinomas of Asian subjects have shown that ras gene mutations can occur in up to 35% of cases. Several explanations might underlie these discrepant results. In contrast with Asian investigators, we only studied early gastric carcinomas. As early gastric carcinoma is thought to precede advanced gastric carcinoma during gastric carcinogenesis, it might well explain the disparity between our results and theirs. European-American studies on advanced gastric carcinomas, however, have also reported ras gene mutations to be very rare or even absent.¹²⁻¹⁴ Interestingly, considerable differences in ras gene mutational spectrum between non-small cell lung cancers in white and Asian subjects³⁷⁻³⁸ have been found. The contrasting findings of ras gene mutations in gastric carcinomas in white and Asian subjects might therefore also relate to differences in patient groups studied, assuming racial differences or geographical differences, or both, in carcinogenic exposure. Further evidence for this assumption is the finding that pancreatic carcinomas of European subjects show considerable geographical differences in mutational spectrum of the Ki-ras gene.³⁹ Finally, as no mutations at all were found in our study, technical caveats should be considered as well. It is well known that DNA isolated from formalin fixed tissues is usually partially degraded into smaller fragments. The extent of degradation is influenced by many factors, for example, the time between removal of the tissue and fixation, the duration of the fixation step, and the type of fixative used. The average length of DNA extracted from (formalin) fixed tissues, however, is usually in the range of 100–500 nucleotides.⁴⁰ As only small DNA fragments need to be amplified for the detection of point mutations in the ras genes, the effect of DNA degradation should be regarded as of minor importance only for the detection of ras gene mutations, in contrast with, for example, mutation analysis of the p53 gene. Moreover, all cases tested positive for the wild-type sequence of the ras genes. As such, the positive wild-type signals served as internal controls for DNA extraction, amplification, and hybridisation procedures in each case. Therefore, in view of the above and in view of the sensitivity of our assays in combination with the fact that the tumour cells constituted at least 50% of the total cell population per case, we strongly feel that the absence of ras gene mutations in our series is genuine and not the result of technical flaws.

Immunohistochemical studies on p21^{ras} expression in gastric carcinomas have reported increased expression of p21^{ras} proteins, especially in intestinal-type carcinomas compared with diffuse-type carcinomas.⁴¹⁻⁴⁵ Moreover, it was found that the normal mucosa surrounding diffuse-type tumours, but not surrounding intestinal-type tumours, showed increased expression of p21^{ras} proteins. Interestingly, intestinal metaplasia and dysplasia, surrounding intestinal-type carcinomas, also showed increased expression

of p21^{ras} proteins.⁴³ It was suggested that these findings supported a role for the ras genes in gastric carcinogenesis and supported the concept of a different carcinogenic pathway of both Lauren types. These suggestions are in contrast with our own findings. Various explanations may account for this apparent discrepancy. Firstly, it should be noted that increased p21^{ras} protein expression in tumours does not necessarily indicate that the levels of the p21^{ras} protein(s) have transforming capability. It is noteworthy that immunohistochemical studies especially can be classified as semi-quantitative at best. Secondly, increased p21^{ras} expression is not necessarily synonymous with malignant phenotype, as it has also been seen in regenerating rat liver and in regenerating epithelium adjacent to peptic ulceration.⁴²⁻⁴⁶ Presumably, a moderate increase in ras gene expression is therefore related to active cell proliferation rather than to malignant phenotype. Thirdly, although overexpression of wild-type p21^{ras} has been implicated in malignant transformation,²⁻⁴⁷⁻⁴⁸ this mechanism is generally considered to be a rare event in tumorigenesis. Fourthly, inherent pitfalls of most p21^{ras} antibodies hamper a correct interpretation of immunohistochemical studies.⁴⁹ Therefore, in view of the above and in view of the absence of activating ras gene mutations in our series, we feel that ras gene abnormalities in all probability do not contribute to gastric carcinogenesis and in all probability do not contribute to the concept of a different pathogenesis of both Lauren types.

To date, the role of *H. pylori* in gastric carcinogenesis is a topic of great interest. Whereas many studies have been published that suggest a role for *H. pylori* in this process, no studies so far have reported on the difference in molecular make up, if any, between *H. pylori* positive and *H. pylori* negative gastric carcinomas. A pitfall of retrospective studies in classifying carcinomas into *H. pylori* positive and *H. pylori* negative cases is that extensive intestinal metaplasia formation, particularly in the course of the carcinogenic process of intestinal-type carcinomas may have led to the disappearance of the micro-organism in the stomach, resulting in an underestimation of its prevalence. Moreover, previous use of antibiotics may also have confounded results. Nevertheless, as ras gene mutations were completely absent in our series, it may be assumed that *H. pylori* positive and *H. pylori* negative gastric carcinomas cannot be discriminated on the basis of ras gene mutational analysis.

Despite the excellent prognosis of surgically treated early gastric carcinomas compared with advanced gastric carcinomas, the metastatic potential and postoperative five year survival rate of early gastric carcinomas seem to be correlated with the type of growth pattern. In particular, the penetrating A type, in contrast with the other growth types, has been reported to have a high propensity for blood vessel invasion and lymph node metastasis and to have the worst prognosis after surgery because of early postoperative hepatic metastasis.²⁶ In a related study we showed that this apparent

difference in clinical or biological behaviour, or both, could not be explained on the basis of a difference in nuclear p53 protein accumulation.⁵⁰ This study shows that ras gene mutations do not underlie these clinical findings either.

In conclusion, activating point mutations at codons 12, 13, and 61 of the Ha-, Ki-, and N-ras genes do not play a part in the development of (early) gastric carcinomas of white subjects irrespective of Lauren type. Moreover, these ras gene mutations do not underlie the differences in clinical or biological behaviour, or both, of early gastric carcinomas with different types of growth pattern. Finally, *H. pylori* positive and *H. pylori* negative gastric carcinomas cannot be discriminated by ras gene mutational analysis.

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