Telomere erosion is independent of microsatellite instability but related to loss of heterozygosity in gastric cancer

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Abstract
AIM  To correlate the length of the telomere to microsatellite instability (MSI) and loss of heterozygosity (LOH) of APC, MCC and DCC genes in gastric carcinomas.

METHODS  Telomeric restriction fragment (TRF) length of gastric cancer was measured with Southern blot. LOH of APC, MCC and DCC genes, microsatellite instability (MSI) and frameshift mutation of hMSH6, TGF-βRI and BAX genes were analyzed by PCR-based methods.

RESULTS  Sixty-eight cases of sporadic gastric carcinoma were studied for MSI using five microsatellite markers. MSI in at least one locus was detected in 17 (25%) of 68 tumors analyzed. Frameshift mutations of hMSH6, TGF-βRI and BAX were detected in 2, 6 and 3 of gastric carcinomas respectively showing high MSI (≥2 loci, n = 8), but none was found in those showing low MSI (only one locus, n = 5) or MSS (tumor lacking MSI or stable, n = 51). Thirty-five cases, including all high MSI and low MSI, were studied for TRF. The mean TRF length was not correlated with clinicopathological parameters. No association was observed between TRF length and MSI or frameshift mutation. On the contrary, LOH at the DCC locus was related to telomere shortening (P < 0.01). This tendency was also observed in APC and MCC genes, although there was no statistical significance.

CONCLUSION  The development of gastric cancer can arise through two different genetic pathways. In high MSI gastric cancers, defective mismatch repair allows mutations to accumulate and generate the high MSI phenotype. In gastric cancers showing either low MSI or MSS, multiple deletions may represent the LOH pathway. Telomere erosion is independent of high MSI phenotype but related to the LOH pathway in gastric cancer.

Subject headings  gastric cancer; telomere restriction fragment; microsatellite instability; loss, heterozygosity

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INTRODUCTION  Eukaryotic linear chromosomes are capped by a special structure known as the telomere. In vertebrates, telomeres consist of several kilobases of tandem TTAGGG repeats bound by the related telomere-specific proteins, TRF1 and TRF2[11]. TRF1 regulates telomere length[11] and TRF2 maintains telomere integrity[16]. In most human cells, telomeres shorten with each cell division due to the incomplete replication of linear DNA molecules and the absence of telomere-elongating mechanisms[15]. It has been reported that telomeric repeats are often reduced in various human tumors and that telomere length is associated with DNA damage mechanism[16]. Telomeres also prevent the activation of DNA-damage check-points[12]. Thus, without these essential structures, the chromosome is unstable.

MSI represents an important new form of genetic alteration characterized by widespread instability in repetitive nucleotide sequences. MSI has been found in the majority of tumors associated with hereditary non-polyposis colorectal cancer (HNPCC)[13,14] in which germ-line mutation occurs within the mismatch repair genes hMSH2, hMLH1, hPMS2 or hMSH6[15,17]. Mutations of the transforming growth factor type II receptor gene (TGF-βRII) and BAX gene are strongly correlated with MSI[18-20]. MSI is also a distinctive feature in about 10%-15% of sporadic colorectal tumors and to a varying degree in tumors of other organs, including the stomach[7,21-24]. Although alterations of telomerase activity have been reported in MSI and microsatellite stable (MSS) tumors[25,26], less clear, however, the relevance of progressive telomere shortening as a potential factor in MSI is less clear. The aim of the present study is to correlate telomere status with MSI and loss of heterozygosity (LOH) of APC, MCC and DCC genes in gastric carcinomas.

MATERIAL AND METHOD  Sixty-eight cancer and corresponding normal tissues were obtained from surgically resected gastric carcinoma patients in our hospital. Each specimen was frozen immediately and...
stored at -80°C until analyzed. A 5μm section was cut from each tissue and stained with hematoxylin/eosin in order to ascertain whether the cancer cells in tissues or not. Genomic DNA was isolated by standard proteinase-K digestion and phenol-chloroform extraction protocols. Of the 68 patients with gastric cancer, 45 were men and 23 were women with an age range of 30-76 years (mean age of 56.2 years at diagnosis). None of the patients included in the present series had a family history suggestive of HNPC and had received chemotherapy or radiation therapy.

MSI analyses included five microsatellite markers: BAT25, BAT26, BAT40, D2S123, and D5S346. PCR was performed in 15µL of reaction mixture containing 10nmol·L⁻¹ Tris-HCl (pH 8.3), 50mmol·L⁻¹ KCl, 1.5mmol·L⁻¹ MgCl₂, 200µmol·L⁻¹ each deoxynucleotide triphosphate, 0.5µmol·L⁻¹ of each primer, 0.75unit Ampli Taq polymerase (Perkin-Elmer, Norwalk, CT, USA), and 100ng genomic DNA. The reaction was carried out in a thermal cycler at 94°C for 1min, 55°C-62°C for 1min, and 72°C for 1min, for 35 cycles with an initial denaturation step of 94°C for 5min and a final extension step of 72°C for 10min. The PCR products were then separated on 5% polyacrylamide, 7M urea denaturing gel, and visualized by autoradiography. MSI was defined as the presence of band shift in the tumor DNA that was not present in the corresponding normal DNA. Based on the number of mutated MSI markers in each tumor, carcinomas were characterized as high MSI if they manifested instability at two or more markers, low MSI if unstable at only one marker, and MSS if they showed no instability at any markers (microsatellite stable) [27-29].

Figure 1 MSI in gastric cancer using 5 microsatellite loci (BAT-25, BAT-26, BAT-40, D2S123, and D5S346). Arrows indicate variant conformers. (N: normal DNA pattern; T: tumor specimens containing variant conformers representing MIS)

Figure 2 Frameshift mutations of hMSH6 TGF-beta RII and BAX genes in gastric cancers. Arrows indicate conformational variants associated with frameshift mutations. (N: normal DNA; T: tumor DNA)

Table 1 Relationship between MSI status and frameshift mutation

| MSI status | No. of cases | Frameshift mutations |
|------------|--------------|----------------------|
|            |              | MSH6 | TGF-beta RII | BAX |
| MSI-H      | 8            | 2    | 6           | 3   |
| MSI-L      | 9            | 0    | 0           | 0   |
| MSS        | 51           | 0    | 0           | 0   |
Thirty-five gastric cancers, including all 8 with high MSI and 9 with low MSI, were examined for alteration in TRF length using the Southern blot technique. Since the signal of telomeric repeats is detected as a smear (Figure 3), we visually determined the peak of signal, which was confirmed using a densitometer. When the TRF length in the tumor was more than 20% shorter or longer than that in corresponding normal tissues, we classified the TRFs as shortened or elongated, as previously described[38]. Compared with the corresponding normal mucosa, 20 (57.1%) had considerable shortening (<80% of corresponding normal tissues), 12 (34.3%) had approximately the same length (between 80%-120%), and 3 (8.6%) showed elongation (>120%). No correlation was found between mean TRF length and clinicopathological parameters in gastric carcinomas. However, a decrease of the mean TRF length with the age was observed except for the group aged from 50 to 59 years. The mean TRF length in the age group of 30 to 39 years was significantly longer than that in the group aged from 60 to 69 years or 70 to 79 years (P<0.05) (Table 2).

We compared the TRF length with MSI and frameshift mutation status. Table 3 shows the association of MSI and frameshift status to the TRF length of 35 gastric cancers. No relationship was observed between TRF length and MSI or frameshift mutation in gastric cancer.

Tissues from 35 patients were also studied for LOH of APC, MCC and DCC. In order to increase the assay sensitivity, three different sites, i.e., M2, M3 and VNTR were used for analysis of LOH at DCC genetic locus. LOH of APC, MCC and DCC were observed in 6 (27.3%) of 22, 7 (36.8%) of 19 and 18 (56.3%) of 32 of informative cases, respectively (Figure 4). The relationship between LOH at various loci and the alteration in TRF length in primary tumors is shown in Table 4. LOH at the DCC locus were associated with telomere shortening (P<0.01). This tendency was also observed in APC and MCC genes, although there was no statistical significance.

Table 2 The relationship between the mean TRF length and clinicopathological parameters

| Parameters       | No. of cases | Mean TRF length (x±s) |
|------------------|--------------|-----------------------|
| Gender           |              |                       |
| Male             | 23           | 3.99±0.63             |
| Female           | 12           | 4.32±1.00             |
| Age(=yrs)        |              |                       |
| 30-39            | 5            | 4.54±0.54             |
| 40-49            | 7            | 4.07±0.60             |
| 50-59            | 11           | 4.39±0.96             |
| 60-69            | 10           | 3.69±0.66             |
| 70-79            | 2            | 3.40±0.07             |
| Size             |              |                       |
| <5cm             | 12           | 3.80±1.20             |
| >5cm             | 23           | 4.06±0.86             |
| Differentiation  |              |                       |
| Well and moderate| 7            | 3.91±0.49             |
| Poor             | 19           | 4.24±0.64             |
| Mucinous         | 9            | 4.02±1.10             |
| Clinical stage   |              |                       |
| I and II         | 21           | 4.26±0.82             |
| III and IV       | 14           | 3.84±0.68             |

*P<0.05 vs the age group of 30 to 39 years.

Table 3 Relationship between alterations of TRF length and MSI status or frameshift mutation

| No. of cases | Alterations in TRF length |
|--------------|---------------------------|
|              | Shortened (n=20) | Normal (n=12) | Elongated (n=3) |
| MSI status   |              |              |               |
| MSI-H        | 8            | 4            | 4             |
| MSI-L        | 9            | 4            | 4             |
| MSS          | 18           | 9            | 7             |
| Frameshift mutation |            |              |               |
| MSH3         | 35           | 1            | 1             |
| TGF β RII    | 35           | 3            | 2             |
| BAX          | 35           | 2            | 1             |

*P<0.01.

Table 4 Relationship between alterations of TRF length and loss of heterozygosity of APC, MCC and DCC genes

| LOH       | Alterations in TRF length (LOH/Informative) |
|-----------|---------------------------------------------|
| APC       | Shortened (n=20) | Normal (n=12) | Elongated (n=3) |
| MCC       | 5/13 | 1/7 | 0/2 |
| DCC       | 6/11 | 1/6 | 0/2 |
|           | 14/18 | 3/11 | 1/3 |

*P<0.01.
In this study, 17(25%) of 68 sporadic gastric cancers had MSI in at least one locus. This finding is similar to previous studies. There is now evidence that MSI cancer comprises distinctive high MSI and low MSI categories. High MSI cancers are distinguished clinicopathologically and in their spectrum of genetic alterations from cancers showing low MSI and MSS cancers. Our previous studies indicated that high MSI gastric cancers often show lower frequency of LOH of APC, MCC and DCC genes than low MSI and MSS. High MSI tumor cells and support the notion that high MSI tumors identify an alternative pathway of tumorigenesis that has been proposed by Vogelstein and co-workers.

In the present study, 35 gastric carcinomas were examined for TRF length using the Southern blot. Telomere shortening was detected in 57.1% of the gastric carcinomas, indicating that telomere erosion may play a role in the gastric carcinoma development. It has been reported that telomere shortening was associated with a short survival and disease recurrent in prostate cancer. In the current study, subdivision of the tumors according to telomere length did not reveal an obvious relationship between the shortening of telomere and tumor size, depth of invasion, node metastasis or clinical stages, indicating a limited role of the telomere shortening in predicting prognosis of gastric carcinomas. This finding is in agreement with the recently published data on renal cell carcinoma and colorectal carcinomas.

TRF reduction was demonstrated in various human malignant tumors. In contrast to these results, it was also found that telomeres in some tumors are similar to, even elongated, as compared with normal tissues. In this study, the mean TRF length was reduced in 20 of 35 tumors, similar in 12 cases and elongated in 3 cases, as compared with the corresponding normal mucosa. The discrepancy in TRF length may be related for several reasons. For tumor samples with TRF of normal length, there are at least three possibilities: 1) The majority of cells in such tumors have not experienced many cell divisions nor experienced critical shortening of telomeric repeats; 2) In most tumor cells, telomerase already activated, elongated the once shortened telomeric repeats back to normal length, as observed in HPV-immortalized epithelial cells in vitro; and 3) The amount of tumor cells also affects TRF analysis. Therefore, with the predominance of normal cells in tumor specimens, telomere loss may be underestimated. We found that the TRF length in tumors was progressively reduced with age, suggesting that patients’ age should be considered in evaluating the TRF lengths of tumor. Finally, end-to-end chromosome fusion observed in some tumors could lead to telomere elongation.

It has been reported that telomerase activity and microsatellite instability are independent events in colorectal carcinogenesis. To our knowledge, this is the first report on the relationship between MSI and telomere length. We did not find any correlation between telomere erosion and MSI, suggesting that the MSI pathway is independent of telomere erosion.

It has been found that chromosomal instability is correlated with telomere erosion and inactivation of G2 checkpoint function in human fibroblasts. Alterations in the TRF length were often associated with LOH of the p53 gene and Rb gene, but less often with mutation of k-ras and p53 genes in lung cancer. We found that LOH at the DCC locus was associated with telomere shortening. Although not statistically significant, LOH at the APC and MCC genetic loci tends to occur in telomere shortened gastric cancers, indicating that telomere erosion may be involved in the LOH pathway.

In summary, our data indicate that telomere shortening may play a role in gastric carcinogenesis. There are at least two distinct genetic instabilities in gastric tumorigenesis: one is the chromosomal instability (or suppressor pathway) and the other is microsatellite instability (or MSI pathway). The former, may include tumors with low MIS as well as MSS and accumulation of loss of tumor suppressor genes such as p53, Rb, APC, MCC and DCC plays an important role in their carcinogenesis; whereas the latter consists of a small subset of gastric cancer with high MSI, defective repair of mismatched bases results in an increased mutation rate at the nucleotide level, and consequent widespread MSI. Telomere shortening may be involved in the LOH pathway but independent of the MSI pathway. Our analysis of TRFs should further provide some clues to the molecular mechanisms underlying the profound genomic instability in the MSI and LOH pathway for gastric carcinoma.

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