Demethylation of MAGE promoters during gastric cancer progression

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Melanoma antigen (MAGE)–encoding genes are expressed in various tumour types via demethylation of their promoter CpG islands, which are silent in all non-neoplastic tissues except for the testis and placenta. The clinicopathological significance of demethylation of MAGE genes in gastric carcinoma is not known. We investigated the promoter methylation status of MAGE-A1 and -A3 in 10 gastric cancer cell lines and in surgical specimens from 84 gastric cancer patients by methylation-specific PCR (MSP). Expression of MAGE-A1 and -A3 in the 10 gastric cancer cell lines was also investigated by RT–PCR. Any correlation between the methylation status of the MAGE promoters and clinicopathological characteristics of the gastric cancer patients was then assessed. Eight of the 10 gastric cancer cell lines showed demethylation of both MAGE-A1 and -A3, and the remaining two cell lines did either of MAGE-A1 or -A3. Expression of MAGE-A1 and -A3 was confirmed in seven and nine of the 10 gastric cancer cell lines, respectively. The MAGE-A1 and -A3 promoters were demethylated in 29% (25 out of 84) and 66% (56 out of 84) of the gastric tumour specimens, respectively. Demethylation of both MAGE-A1 and -A3 promoters (n = 22) was found more frequently in gastric cancer patients in advanced clinical stages (P = 0.0035), and these patients also exhibited a higher incidence of lymph node metastasis (P = 0.0007) compared to those patients without demethylation (n = 25). Furthermore, demethylation patients tended to have a worse prognosis, although this difference was not statistically significant (P = 0.183). Demethylation of MAGE-A1 and -A3 occurs during progressive stages of gastric cancer, and may be associated with aggressive biological behaviour of gastric cancer.

Keywords: gastric cancer; MAGE; demethylation

Epigenetic alterations, including hypermethylation of promoter CpG islands and histone deacetylation of tumour suppressor and tumour-related genes (Choi et al., 2001; Jones and Takai, 2001; Kim et al., 2001; Goodman and Watson, 2002; Jones and Baylin, 2002) as well as global DNA hypomethylation (Eden et al., 2003; Gaudet et al., 2003; Lengauer, 2003), have been recognised as important contributors to carcinogenesis in humans. Global DNA hypomethylation has been observed in carcinomas of the breast, liver, and colon, and is considered to occur in the early stages of tumour development (Goelz et al., 1985; Cravo et al., 1996; Narayan et al., 1998; Lin et al., 2001; Bariol et al., 2003). However, little is known about promoter hypomethylation of specific genes such as oncogenes and growth-related genes, with the exception of the association between demethylation and increased expression of c-abl, c-myc, Ha-ras, and ras (Cheah et al., 1984; Weitzman et al., 1989; Sharrad et al., 1992; Counts and Goodman, 1994).

Human melanoma cells express antigens that are recognised by cytolytic T lymphocytes derived from the blood of tumour-bearing patients or from tumour-infiltrating lymphocytes (Boon et al., 1994). A number of such antigens are encoded by genes of the MAGE family (Van der Bruggen et al., 1991; Lucas et al., 2000). A total of 19 MAGE genes are located on chromosome X (Lucas et al., 2000), and are expressed in other tumours, including gastric cancer, in addition to melanoma (Van der Bruggen et al., 1991; Brasseur et al., 1992; Chambost et al., 1993; Inoue et al., 1995a, b; Gotoh et al., 1998; Takahashi et al., 1998). Although the functions of the various MAGE proteins remain to be elucidated, MAGE gene expression is known to be activated by promoter demethylation in a similar manner as the oncogenes and growth-related genes described above (De Smet et al., 1996). These genes are silent in normal tissues except for the testis and placenta (De Pena et al., 1994; De Smet et al., 1994), and may be targets for future cancer immunotherapies (Marchand et al., 1999; Jang et al., 2001).

In the present study, we investigated the promoter methylation status of MAGE-A1 and -A3, which were the most frequent targets for immunotherapy, in gastric cancers, and analysed the correlation between the MAGE-A1 and -A3 methylation status and clinicopathological parameters of gastric cancer patients, including event-free survival.

MATERIALS AND METHODS

Gastric cancer cell lines

We have investigated 10 gastric cancer cell lines with variable histologies that were cultured under appropriate conditions in our
Primary gastric cancers

In total, 84 pairs of cancerous and noncancerous gastric tissues (51 differentiated and 31 undifferentiated carcinomas; 25 early-stage carcinomas that demonstrated a depth of invasion limited to the submucosa and 57 advanced stage carcinomas) were surgically obtained from 84 gastric cancer patients. These tissues were immediately frozen and stored at −80°C until analysis. All patients received a median of 36.7 months of follow-up care (range, 1–77 months). Signed informed consent was obtained from every patient to allow the use of biological materials for biological studies.

DNA extraction

DNA was extracted from 10 gastric carcinoma cell lines and 84 primary gastric cancers and their corresponding noncancerous gastric tissues with SepaGene (Sanko-Junyaku, Tokyo, Japan).

RNA extraction

Total RNA was isolated from 10 gastric carcinoma cell lines with the TRIZOL reagent (Gibco BRL, Life Technologies, Gaithersburg, MD, USA).

Bisulphite modification and methylation-specific polyacrylamide chain reaction (MSP)

Treatment of DNA samples with sodium bisulphite converts all unmethylated cytosines to uracils and does not affect methylated cytosines. Briefly, 2 μg of genomic DNA were denatured with sodium hydroxide and modified by sodium bisulphite. The samples were then purified using Wizard DNA purification resin (Promega, Madison, WI, USA), treated with NaOH, recovered in ethanol, and resuspended in 30 μl of distilled water. Amplification was achieved in a 20 μl reaction volume containing 2 μl of GeneAmp PCR Gold Buffer (PE Applied Biosystems, Foster City, CA, USA), 1.0 μM MgCl₂, 1 μl each primer, 0.2 mM dNTPs, and 1 U Taq polymerase (AmpliTaq Gold DNA Polymerase, PE Applied Biosystems). After heating at 94°C for 10 min, polymerase chain reaction (PCR) was performed in a thermal cycler (GeneAmp 2400, PE Applied Biosystems) for 35 cycles, each of which consisted of denaturation at 94°C for 30 s, annealing at 54°C for 60 s, and extension at 72°C for 60 s, followed by a final 7-min extension at 72°C. A positive control (Sss-I methylase-treated DNA) and negative control (distilled water without DNA) were included for each amplification. The PCR products were separated on a 6% nondenaturing polyacrylamide gel. The following primer sets were designed for the amplification. The PCR products were separated on a 6% nondenaturing polyacrylamide gel. The following primer sets were designed for the amplification. The PCR products were separated on a 6% nondenaturing polyacrylamide gel. The following primer sets were designed for the amplification. The PCR products were separated on a 6% nondenaturing polyacrylamide gel. The following primer sets were designed for the amplification.
Correlation between demethylation of MAGE promoters and clinicopathological parameters

Gastric cancer patients who exhibited demethylation of both the MAGE-A1 and -A3 promoters (n = 22) were at a more advanced clinical stage (P = 0.0035), and had a higher incidence of lymph node metastasis (P = 0.0007) compared with those who did not have demethylated MAGE-A1 and -A3 promoters (n = 25) (Table 1). Furthermore, patients with demethylated MAGE-A1 and -A3 promoters tended to have a worse prognosis, although this difference was not statistically significant by the log rank test (P = 0.183) (Figure 4). Patients with demethylation of only one of the two promoters exhibited biological features intermediate to those of the other two groups.

Figure 1  Methylation-specific PCR of gastric cancer cell lines. (A) Methylated-sequence-specific PCR of MAGE-A1; (B) Unmethylated-sequence-specific PCR of MAGE-A1; (C) Methylated-sequence-specific PCR of MAGE-A3; (D) Unmethylated-sequence-specific PCR of MAGE-A3; P, positive control; DW, distilled water; SM, size marker. Methylated MAGE-A1 is present in lanes 4 and 7 (A), and demethylated MAGE-A1 is present in all lanes except lane 4 (B). Methylated MAGE-A3 is present in lanes 1–7 and 9 (C), and demethylated MAGE-A3 is present in all lanes except lane 7 (D). Lanes: 1, MKN1; 2, MKN7; 3, MKN28; 4, MKN45; 5, MKN74; 6, KATO-III; 7, KWS-I; 8, TSG11; 9, ECC10; and 10, ECC12.

Figure 2  Reverse transcription–PCR of gastric cancer cell lines; (A) RT–PCR of MAGE-A1; (B) RT–PCR of MAGE-3; (C) RT–PCR of \( \beta \)-actin; SM, size marker. MAGE-A1 mRNA is not present in lanes 4, 7, or 9 (A). MAGE-A3 mRNA is not present in lane 7 (B); \( \beta \)-actin serves as an internal control (C). Lanes: 1, MKN1; 2, MKN7; 3, MKN28; 4, MKN45; 5, MKN74; 6, KATO-III; 7, KWS-I; 8, TSG11; 9, ECC10; and 10, ECC12.

Figure 3  Methylation-specific PCR of primary gastric cancer specimens and their corresponding non-neoplastic gastric tissues; (A) methylated-sequence-specific PCR of MAGE-A1 in gastric cancer specimens; (B) unmethylated-sequence-specific PCR of MAGE-A1 in gastric cancer specimens; (C) methylated-sequence-specific PCR of MAGE-A3 in non-neoplastic gastric tissues; (D) unmethylated-sequence-specific PCR of MAGE-A3 in non-neoplastic gastric tissues; (E) methylated-sequence-specific PCR of MAGE-A3 in gastric cancer specimens; (F) unmethylated-sequence-specific PCR of MAGE-A3 in gastric cancer specimens; (G) methylated-sequence-specific PCR of MAGE-A3 in non-neoplastic gastric tissues; (H) unmethylated-sequence-specific PCR of MAGE-A3 in non-neoplastic gastric tissues; P, positive control; DW, distilled water; SM, size marker. Methylated MAGE-A1 and -A3 is present in all lanes (A, C, E, and G). In gastric cancer specimens, demethylated MAGE-A1 and -A3 are present in lanes 1, 6, and 7 of (B) and in lanes 5–8 of (F), respectively, whereas none of the non-neoplastic gastric tissues exhibit demethylation of MAGE-A1 or -A3 (D and H). Lanes: 1, M244; 2, M245; 3, M246; 4, M248; 5, M251; 6, M254; 7, M256; 8, M257; and 9, M262.
DISCUSSION

MAGE-A1 and -A3 encode tumour-specific antigens that are recognised on melanoma cells by autologous cytolytic T lymphocytes (Van der Bruggen et al., 1991; Boon et al., 1994). These genes are expressed in a significant proportion of tumours of various histological types, but not in normal tissues, except for male germ line cells and placenta (De Plaen et al., 1994; Takahashi et al., 1995). Demethylation of promoter CpG islands in MAGE genes triggers their expression in tumour cells, whereas they are not expressed in cells in which they remain methylated (De Smet et al., 1996). The function of the MAGE peptides are not known, although their tumour-specific expression is clearly of great importance for immunotherapy (Marchand et al., 1999; Nishiyama et al., 2001; Sadanaga et al., 2001). The MAGE-A1 and -A3 peptides are expressed in 67–73% of gastric cancer cell lines (Inoue et al., 1995a; Li et al., 1996). In agreement with these data, we have demonstrated that MAGE-A1 and -A3 mRNA are expressed in 70 and 90% of gastric cancer cell lines, respectively. MAGE-A1 and -A3 mRNA has also been reported to be expressed in approximately 40% of primary gastric cancers (Inoue et al., 1995a, b; Li et al., 1996). However, these previous studies did not verify the methylation status of the MAGE-A1 and -A3 promoter CpG islands. In the present study, we showed that the methylation status of the MAGE-A1 and -A3 promoters almost directly correlated with their expression status in gastric cancer cell lines.

Global DNA hypomethylation is thought to occur during the early stages of tumour development in gastric and other tissues (Goelz et al., 1985; Narayan et al., 1998; Lin et al., 2001; Bariol et al., 2003). Additionally, in pulmonary carcinogenesis, demethylation of the promoter CpG islands of MAGE genes has been observed not only in tumours but also in the adjacent non-neoplastic lung tissues and bronchial epithelia from smokers (Jang et al., 2001). Therefore, MAGE genes may be activated prior to malignant transformation in the lung, possibly by global DNA hypomethylation (Tamura, 2002). However, we have demonstrated that MAGE gene promoters are demethylated more frequently in gastric cancers at advanced clinical stages. Furthermore, demethylation of

| Promoter methylation status | Not altered | One demethylated promoter | Two demethylated promoters |
|----------------------------|------------|---------------------------|----------------------------|
| Number of patients         | 25         | 37                        | 22                         |
| Age (mean) (Years)         | 66.8       | 64.6                      | 69.1                       |
| Gender                     |            |                           |                            |
| M                         | 20         | 25                        | 19                         |
| F                         | 5          | 12                        | 3                          |
| Unknown                    | 0          | 0                         | 0                          |
| Stage                      |            |                           |                            |
| Early                      | 15         | 11                        | 4                          |
| Advanced                   | 10         | 26                        | 18                         |
| Unknown                    | 0          | 0                         | 0                          |
| Histological differentiation|            |                           |                            |
| Differentiated             | 16         | 24                        | 12                         |
| Undifferentiated           | 9          | 13                        | 10                         |
| Unknown                    | 0          | 0                         | 0                          |
| Location                   |            |                           |                            |
| Lower                      | 8          | 15                        | 10                         |
| Middle                     | 10         | 15                        | 5                          |
| Upper                      | 5          | 4                         | 6                          |
| Unknown                    | 2          | 3                         | 1                          |
| Lymph node metastasis      |            |                           |                            |
| Present                    | 7          | 21                        | 17                         |
| Absent                     | 18         | 16                        | 5                          |
| Unknown                    | 0          | 0                         | 0                          |

NS = not significant by Fisher’s exact probability test.

Figure 4 Methylation status and survival curve for gastric cancer patients. Patients in the ‘two demethylated promoter’ group tended to have a worse prognosis than patients in the ‘not altered’ group (P = 0.183). Patients in the ‘one demethylated promoter’ group exhibited an intermediate survival time between the two.
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MAGE-A1 and -A3 is quite rare in non-neoplastic gastric tissues of gastric cancer patients. In a separate study, we have confirmed that demethylation of MAGE-A1 and -A3 was also very rare in various organs obtained at autopsies, from various age groups (data not shown). Therefore, we hypothesise that demethylation of MAGE genes occurs during progressive stages of gastric carcinogenesis, probably after global DNA hypomethylation. Promoter CpG islands of several tumour suppressor and tumour-related genes are frequently methylated in both neoplastic and non-neoplastic gastric epithelia (Tamura, 2002; Waki et al, 2002). Hypermethylation of different genes increases with age in different organs (Waki et al, 2003). These results suggest that hypermethylation of promoter CpG islands occurs very early in gastric carcinogenesis, in contrast to demethylation of MAGE gene promoters.

Several studies have analysed MAGE gene expression Inoue et al, 1995a, b; Cravo et al, 1996; Li et al, 1996; Sadanaga et al, 2001), but none have evaluated the demethylation status of their promoters in gastric cancer. Inoue et al (1995a, b) detected MAGE expression in about 40% of primary gastric cancers, but failed to find any significant correlation between MAGE expression and clinicopathological parameters (Inoue et al, 1995a, b). In the present study, demethylation of either MAGE-A1 or -A3 was not significantly correlated with clinicopathological parameters, but demethylation of both genes significantly correlated with advanced clinical stage and lymph node metastasis. Furthermore, we have noticed that patients with tumours showing demethylation of both MAGE-A1 and -A3 tend to have a worse prognosis, although this difference was not statistically significant. In contrast, hypermethylation of the hMLH1 gene promoter is a marker of a better prognosis (Yamamoto et al, 1999). No correlation has been observed between demethylation of the MAGE genes and hypermethylation of hMLH1 or p16 (data not shown).

In summary, demethylation of the MAGE-A1 and -A3 promoters frequently occurs during progressive stages of gastric carcinogenesis and may be associated with aggressive biological behaviour of gastric cancer.

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