Different Susceptibility of Each L-myc Genotype to Esophageal Cancer Risk Factors

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To understand the relationship between the L-myc genotypes and esophageal cancer risk, a polymerase chain reaction-based restriction fragment length polymorphism analysis was performed on 91 Japanese patients with esophageal cancer and 241 non-cancer outpatients. No significant difference in the distribution of genotypes was observed between patients and controls; 18.7% LL genotype, 56.0% LS and 25.3% SS among patients, and 24.5%, 55.6% and 19.9%, respectively, among controls. Frequency of the s-allele in patients (0.533) was slightly higher than in controls (0.477), but the difference was not statistically significant. However, the odds ratios (ORs) for smoking or heavy drinking were markedly higher in SS and LS genotypes than in LL genotype; age-sex-adjusted ORs for smoking was 7.57 in the SS genotype, 6.40 in the LS genotype and 1.77 in the LL genotype. Age-sex-adjusted ORs for heavy drinking were 19.78, 18.20 and 7.40, respectively. The age-sex-adjusted ORs for both factors combined were 12.77, 18.45 and 1.44, respectively. These results suggested that the L-myc polymorphism might modify the effects of lifestyle factors on esophageal cancer risk.

Key words: Esophageal cancer — L-myc polymorphism — Risk factor — Lifestyle

The L-myc gene, belonging to the Myc family, was first isolated from a small cell lung cancer and located on chromosome 1p32.1) Genomic DNA within the L-myc locus shows an EcoRI RFLP defined by two alleles with the 10 kb fragment (l-allele) and the 6.6 kb fragment (s-allele), which gives three genotypes, LL, LS and SS.2) This polymorphism can also be detected by EcoRI digestion of the PCR fragment including the polymorphic site.3, 4) Though no functional differences between l- and s-alleles are known, the association of s-allele with tumor susceptibility was reported in non-Hodgkin’s lymphoma,5) gastric cancer,6, 7) hepatocellular carcinoma9) and sarcoma.9) It was also reported that lung cancer patients with s-allele exhibited a much higher incidence of metastasis.10–12) On the other hand, no associations were reported between the L-myc genotype and susceptibility to renal cancer,13) oral cancer,14) gastric cancer,6, 15) breast cancer,6) lung cancer,16, 17) or bladder cancer.18) Therefore the relationship between cancer susceptibility and the L-myc polymorphism is not yet well established.

To date, only one study has been reported on the relationship between susceptibility to esophageal cancer and the L-myc polymorphism, showing that esophageal cancer risk was increased in the SS genotype.19) Since opposite results were reported for the relationship between the susceptibility to some types of cancer and the L-myc polymorphism as described above, further studies are also required on esophageal cancer. In the present study, we assessed the relationship between the L-myc genotype and esophageal cancer risk. The risk of lifestyle factors was also examined in each genotype, since some lifestyle factors such as smoking and drinking are well-known risk factors for esophageal cancer.20, 21)

MATERIALS AND METHODS

Subjects Blood samples were collected from 91 esophageal cancer patients diagnosed between 1984 and 2000 (cases) and 241 non-cancer volunteers among gastroscopy examinees (controls) at the Aichi Cancer Center Hospital. Effects of lifestyle factors were analyzed with questionnaires obtained from 91 cases and 241 controls. Age and sex distributions in cases and controls are shown in Table I. Consistent with a previous report,21) the majority of cases in our study were males. All the controls were used.
for case-control analysis without age-sex-matching, since it was demonstrated to be a better approach.22) Informed consent was obtained from all subjects in this study.

**Analysis of L-myc genotype by PCR-RFLP**

Genomic DNA was isolated from peripheral blood cells using the QIAamp DNA Blood Mini Kit (QIAGEN Inc., Hilden, Germany). For specific amplification of intron 2 of the L-myc locus including the EcoRI polymorphic site, PCR was performed according to Shibuta et al.7) with the upstream primer, 5'-ACGGCTGGTGGAGTGGTAGA-3', and the downstream primer, 5'-AAGCTTGAGCCCTTTGTCA-3'. The PCR was performed with 30 cycles of denaturation at 94°C for 20 s, annealing at 55°C for 20 s and extension at 72°C for 30 s. Amplified fragments were then digested with EcoRI and separated by electrophoresis on 2% agarose gel.

**Questionnaire and data collection**

The questionnaire elicited information about subjects' age, sex, smoking status and alcohol consumption. For analyzing the risk of smoking, only current smokers were included in the category. For analyzing the risk of heavy drinking, people who drink alcohol 5 days or more a week and 50 ml ethanol or more per day were included in the category.

**Statistical analysis and risk assessment for lifestyle**

For statistical analysis of genotype distribution, the \( \chi^2 \) test was performed. The age (as a continuous variable)- and sex-adjusted ORs and 95%CI were estimated for smoking status and alcohol consumption, using an unconditional logistic regression model. The model was also applied to the estimation of age-sex-adjusted interaction terms of genotype with smoking and/or heavy drinking, i.e., the ratio of the ORs for the lifestyle factors by genotype. The 'logistic' procedure provided by STATA version 6.0 (STATA Corp., College Station, TX) was used for the calculations. Adjustments for multiple comparisons were not conducted because this is an exploratory study.

**RESULTS**

**Distribution of L-myc genotype in cases and controls**

The EcoRI digestion of PCR products clearly revealed the l-allele band (613 bp) and the s-allele band (481 bp). Representative results are shown in Fig. 1, and the results are summarized in Table II. The observed frequency of each genotype in cases and controls fitted the Hardy-Weinberg equilibrium (\( \chi^2 = 1.44, P = 0.230 \) and \( \chi^2 = 3.15, P = 0.076 \), respectively). No statistically significant difference in the distribution of the genotypes was found between cases and controls (\( \chi^2 = 1.86, \text{degree of freedom}=2, P = 0.39 \)). The age-sex-adjusted ORs for LS and SS genotypes were not significantly different from that for LL genotype.

**ORs for smoking and heavy drinking according to L-myc genotype**

We analyzed the effects of smoking and/or heavy drinking in each genotype group, as well as the whole subjects. As summarized in Table III, the age-sex-adjusted ORs for smoking and/or heavy drinking were significantly high. Subgroup analyses according to the L-myc genotypes showed that the ORs in the SS and LS genotypes for smoking and/or heavy drinking were signifi-
The age-sex-adjusted ORs for smoking or heavy drinking were significantly high, while the ORs in the LL genotype for smoking and for smoking and heavy drinking were not. Though not statistically significant, marked differences in the ORs between LL and LS/SS genotypes were observed for smoking or heavy drinking, especially for smoking, the OR in LS/SS genotypes being four times higher than that in the LL genotype; the age-sex-adjusted interaction was 2.56 (95%CI 0.98–6.66). Meanwhile, that for heavy drinking was 2.5 times higher than that in the LL genotype, and the corresponding interaction between LL and LS/SS genotypes was 1.80 (95%CI 0.58–5.58). When both smoking and heavy drinking were added to the logistic model, the ORs for smoking or heavy drinking were similar for the whole subjects and each genotype, except that the OR in the SS genotype for smoking lost significance. The OR for both factors combined in LS/SS genotypes was 9–13 times higher than that in the LL genotype. The age-sex-adjusted interaction between LL and LS/SS genotypes was statistically significant (7.20, 95%CI 1.57–32.94).

**DISCUSSION**

Several studies were reported on the relationship between the **L-myc** polymorphism and susceptibility to a variety of cancers after the polymorphism was identified. Some of these studies showed a positive relationship between **L-myc** genotypes and susceptibility to some types of cancer,7, 9, 19) and metastasis,6, 11, 12) but other studies found no relationship between **L-myc** genotypes and susceptibility to the same and other types of cancer6, 13–18, 23) and metastasis6, 13, 15) This suggests that further studies are required to clarify the relationship between **L-myc** genotypes and susceptibility to cancer.

In our results, the frequencies of the three genotypes in esophageal cancer patients and controls fitted the Hardy-Weinberg equilibrium. The genotype distribution in cases was not significantly different from that in controls, although the s-allele was more frequent in the patients. Shibuta et al.19) recently reported a significantly different distribution of the genotype and significantly more s-allele in esophageal cancer patients, though the OR for LS/SS genotypes relative to LL genotype was not significant (OR=2.90, 95%CI 0.54–6.54). The allele frequency in this study was not significant (χ²=1.65, P=0.200), but both studies showed a similar role of the s-allele.

The age-sex-adjusted OR for smoking was 4.99 and the age-sex-drinking status-adjusted OR was 4.22, while the corresponding ORs for heavy drinking were 13.95 and 27.86, respectively (Table III). However, the interaction term was not significant because of the small number of patients. The OR for smoking and heavy drinking combined was 9.19. The OR was markedly high in the LS and SS genotypes, while the LL genotype had a relatively small OR. The interaction term was significant. To our knowledge, this is the first report on the interaction of the **L-myc** polymorphism with smoking and drinking. To confirm this result, a further study with a much larger sample of patients is essential.

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### Table III. Age-sex-adjusted ORs and 95% CIs of Smoking and Heavy Drinking According to **L-myc** Genotype

| Genotype | Casesa | Controlsb | ORc | 95% Clc | Pd | OR | 95% Cl | Pe |
|----------|--------|---------|-----|--------|----|----|--------|----|
| **Smoking** |       |         |     |        |    |    |        |    |
| total    | 33/58  | 186/55  | 4.99| 2.76–9.02| <0.001| 4.22<sup>1)</sup> | 2.15–8.30<sup>1)</sup> | <0.001<sup>1)</sup> |
| LL       | 8/9    | 39/20   | 1.77| 0.54–5.75| 0.345| 1.38| 0.37–5.11| 0.631|
| LS       | 18/33  | 111/23  | 6.40| 2.74–14.95| <0.001| 7.49| 2.65–21.16| <0.001|
| SS       | 7/16   | 36/12   | 7.57| 1.91–30.03| 0.004| 3.26| 0.65–16.44| 0.152|
| **Heavy drinking** |       |         |     |        |    |    |        |    |
| total    | 30/61  | 215/26  | 13.95| 6.99–27.86| <0.001| 12.26<sup>1)</sup> | 5.97–25.19<sup>1)</sup> | <0.001<sup>1)</sup> |
| LL       | 7/10   | 52/7    | 7.40| 1.86–29.43| 0.004| 7.08| 1.75–28.57| 0.006|
| LS       | 16/35  | 121/13  | 18.20| 6.52–50.81| <0.001| 20.40| 6.52–63.81| <0.001|
| SS       | 7/16   | 42/6    | 19.78| 4.26–91.84| <0.001| 13.17| 2.64–65.71| 0.002|
| **Smoking and heavy drinking** |       |         |     |        |    |    |        |    |
| total    | 51/40  | 226/15  | 9.19| 4.42–19.12| <0.001|         |         |    
| LL       | 13/4   | 53/6    | 1.44| 0.33–6.34 | 0.630|         |         |    
| LS       | 28/23  | 129/5   | 18.45| 5.43–62.61| <0.001|         |         |    
| SS       | 10/13  | 44/4    | 12.77| 3.13–52.18| <0.001|         |         |    |

a) “Non-current smokers”/“current smokers” for smoking and “those who drink 50 ml or over ethanol and 5 days or more a week”/“the others” for heavy drinking.

b) Age-sex-adjusted.
c) Age-sex- and smoking status-adjusted.
d) Age-sex- and smoking status-adjusted.
Since alcohol and tobacco consumption differ greatly among local areas in Japan,25, 26 it is possible that the proportion of smokers and/or heavy drinkers among the subjects was larger in the study by Shibuta et al.19 than ours, though they did not mention the frequencies of smokers and heavy drinkers among their subjects. If so, populations with s-allele might exhibit a higher susceptibility to esophageal cancer than those without s-allele.

It is unclear why the risks of smoking and heavy drinking are different among the genotypes, since functional differences between l- and s-alleles have not been reported. Members of the Myc gene family are often activated in a variety of human tumors, such as the L-myc gene amplification in human small cell lung cancer,1) the N-myc gene amplification in neuroblastoma,27) and the c-myc gene overexpression in breast cancer cells.28) Though the relationship between the level of L-myc expression and genotypes is not clear, it is possible that a different level of expression might be caused by each allele. Alternatively, each allele of the L-myc gene may be associated with polymorphism of an unknown gene located in the vicinity of the L-myc locus that influences susceptibility to esophageal cancer. Furthermore, alternative splicing of the L-myc transcript was reported, and could play a different role.2 29) It is also possible that each allele of the L-myc gene influences this alternative splicing. In any case, to elucidate the molecular mechanism of the relationship between each L-myc genotype and the risk associated with lifestyle factors, further molecular analysis of the L-myc gene function must be performed.

It would be of interest to know whether the differences in the ORs for smoking and/or heavy drinking according to the L-myc genotype are also observed in other smoking and/or heavy drinking related cancers, such as head and neck cancer and lung cancer. If the genotypes enhancing the effects of smoking and/or alcohol are identified, more individualized recommendations to quit smoking and/or to reduce alcohol intake will become possible with a beneficial impact on cancer prevention. We are now conducting case-control studies of the possible relationship between the L-myc polymorphism and risk factors among these cancers.

ACKNOWLEDGMENTS

We are grateful to Dr. N. Uchida for his helpful comments on this manuscript. This work was supported in part by the Bristol-Myers Squibb Biomedical Research Grants Program.

(Received December 21, 2000/Revised April 20, 2001/Accepted May 8, 2001)

REFERENCES

1) Nau, M. M., Brooks, B. J., Battey, J., Sausville, E., Gazdar, A. F., Kirsch, I. R., McBride, O. W., Bertness, V., Hollis, G. F. and Minna, J. D. L-myc, a new myc-related gene amplified and expressed in human small cell lung cancer. Nature, 318, 69–73 (1985).

2) Kaye, F., Battey, J., Nau, M., Brooks, B., Seiffer, E., De Greve, J., Birrer, M., Sausville, E. and Minna, J. Structure and expression of the human L-myc gene reveal a complex pattern of alternative mRNA processing. Mol. Cell. Biol., 8, 186–195 (1988).

3) Taylor, J. A., Bell, D. A. and Nagorney, D. L-myc protooncogene alleles and susceptibility to hepatocellular carcinoma. Int. J. Cancer, 54, 927–930 (1993).

4) Weston, A., Ling-Cawley, H. M., Caporaso, N. E., Bowman, E. D., Hoover, R. N., Trump, B. F. and Harris, C. C. Determination of the allelic frequencies of an L-myc and a p53 polymorphism in human lung cancer. Carcinogenesis, 15, 583–587 (1994).

5) Crossen, P. E., Morrison, M. J. and Colls, B. M. Increased frequency of the S allele of the L-myc oncogene in non-Hodgkin’s lymphoma. Br. J. Cancer, 69, 759–761 (1994).

6) Ishizaki, K., Kado, M., Ikenaga, M., Honda, K., Ozawa, K. and Toguchida, J. Correlation of L-myc genotypes to metastasis of gastric cancer and breast cancer. J. Natl. Cancer Inst., 82, 238–239 (1990).

7) Shibuta, K., Mori, M., Haraguchi, M., Yoshikawa, K., Ueo, H. and Akiyoshi, T. Association between restriction fragment length polymorphism of the L-myc gene and susceptibility to gastric cancer. Br. J. Cancer, 85, 681–684 (1998).

8) Hisch, L. L., Huang, R. C., Yu, M. W., Chen, C. J. and Liaw, Y. F. L-myc, GST M1 genetic polymorphism and hepatocellular carcinoma risk among chronic hepatitis B carriers. Cancer Lett., 103, 171–176 (1996).

9) Kato, M., Toguchida, J., Honda, K., Sasaki, M. S., Ikenaga, M., Sugimoto, M., Yamaguchi, T., Kotoura, Y., Yamamuro, T. and Ishizaki, K. Elevated frequency of a specific allele of the L-myc gene in male patients with bone and soft-tissue sarcomas. Int. J. Cancer, 45, 47–49 (1990).

10) Kawashima, K., Shikama, H., Imoto, K., Izawa, M., Naruke, T., Okabayashi, K. and Nishimura, S. Close correlation between restriction fragment length polymorphism of the L-MYC gene and metastasis of human lung cancer to the lymph nodes and other organs. Proc. Natl. Acad. Sci. USA, 85, 2353–2356 (1988).

11) Kawashima, K., Nomura, S., Hirai, H., Fukushima, S., Karube, T., Takeuchi, K., Naruke, T. and Nishimura, S. Correlation of L-myc RFLP with metastasis, prognosis and multiple cancer in lung-cancer patients. Int. J. Cancer, 50, 557–561 (1992).

12) Zborovskaya, I., Gasparian, A., Kitaeva, M., Polotsky, B., Tupizin, N., Machaladze, Z., Gerasimov, S., Shutman, M., Jakubovskaya, M., Davidov, M. and Tatosyan, A. Simultaneous detection of genetic and immunological markers in non-small cell lung cancer: prediction of metastatic poten-
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tial of tumor. Clin. Exp. Metastasis, 14, 490–500 (1996).
13) Kakehi, Y. and Yoshida, O. Restriction fragment length polymorphism of the L-myc gene and susceptibility to metastasis in renal cancer patients. Int. J. Cancer, 43, 391–394 (1989).
14) Saranath, D., Panchal, R. G., Nair, R., Mehta, A. R., Sanghavi, V. and Deo, M. G. Restriction fragment length polymorphism of the L-myc gene in oral cancer patients. Br. J. Cancer, 61, 530–533 (1990).
15) Mironov, N. M., Aguelon, A.-M., Potapova, G. I., Gorbunov, O. V., Klimenkov, A. A. and Yamasaki, H. L-myc allele polymorphism and prognosis for metastases in Russian gastric cancer patients. Eur. J. Cancer, 30A, 1732 (1994).
16) Ge, H., Lam, W. K., Lee, J., Wong, M. P., Yew, W. W. and Lung, M. L. Analysis of L-myc and GST1 genotypes in Chinese non-small cell lung carcinoma patients. Lung Cancer, 15, 355–366 (1996).
17) Tamai, S., Sugimura, H., Caporaso, N. E., Resau, J. H., Trump, B. F., Weston, A. and Harris, C. C. Restriction fragment length polymorphism analysis of the L-myc gene locus in a case-control study of lung cancer. Int. J. Cancer, 46, 411–415 (1990).
18) Ejarque, M. J., Vicente, M., Bernues, M., Oliver, A., Vicente, J., Capella, G., Lluis, F. and Chechile, G. Restriction fragment length polymorphism of the L-myc gene is not a prognostic factor in bladder cancer patients. Br. J. Cancer, 79, 1855–1858 (1999).
19) Shibuta, K., Inoue, H., Sato, K., Matsuyama, A., Ueo, H., Tanaka, Y., Mafune, K., Barnard, G. F. and Mori, M. L-myc restriction fragment length polymorphism in Japanese patients with esophageal cancer. Jpn. J. Cancer Res., 91, 199–203 (2000).
20) Vizcaino, A. P., Parkin, D. M. and Skinner, M. E. G. Risk factors associated with oesophageal cancer in Bulawayo, Zimbabwe. Br. J. Cancer, 72, 769–773 (1995).
21) Garidou, A., Tzonou, A., Lipworth, L., Signorello, L. B., Kalapothaki, V. and Trichopoulos, D. Life-style factors and medical conditions in relation to esophageal cancer by histologic type in a low-risk population. Int. J. Cancer, 68, 295–299 (1996).
22) Hamajima, N., Hirose, K., Inoue, M., Takezaki, T., Kuroishi, T. and Tajima, K. Case-control studies: matched controls or all available controls? J. Clin. Epidemiol., 47, 971–975 (1994).
23) Togu, A. V., Suspects, E. N., Grigoriev, M. Y., Ilyushik, E. S., Karpova, M. B., Hanson, K. P. and Imyanitov, E. N. L-myc polymorphism in cancer patients, healthy blood donors and elderly, tumor-free individuals in Russia. Int. J. Cancer, 85, 747–750 (2000).
24) Castellsague, X., Munoz, N., De Stefani, E., Victora, C. G., Castelletto, R., Rolon, P. A. and Quintana, M. J. Independent and joint effects of tobacco smoking and alcohol drinking on the risk of esophageal cancer in men and women. Int. J. Cancer, 82, 657–664 (1999).
25) Hashimoto, T., Fujita, Y., Ueshima, H., Kagamimori, S., Kasamatsu, T., Morioka, S., Mikawa, K., Naruse, Y., Nakagawa, H. and Haru, N. Urinary sodium and potassium excretion, body mass index, alcohol intake and blood pressure in three Japanese populations. J. Hum. Hypertens., 3, 315–321 (1989).
26) Ministry of Health and Welfare. “Smoking and Health: Report on Smoking and Health Problems,” Ver. 2, pp. 278 (1993). Japan Health Promotion and Fitness Foundation. Hokendouzinsha, Tokyo (in Japanese).
27) Brodeur, G. M., Seeger, R. C., Schwab, M., Varmus, H. E. and Bishop, J. M. Amplification of N-myc in untreated human neuroblastomas correlates with advanced disease stage. Science, 224, 1121–1124 (1984).
28) Schoenenberger, C. A., Andres, A. C., Groner, B., Van Der Valk, M., Lemeur, M. and Gerlinger, P. Targeted c-myc gene expression in mammary glands of transgenic mice induces mammary tumours with constitutive milk protein gene transcription. EMBO J., 7, 169–175 (1988).
29) De Greve, J., Battey, J., Fedorko, J., Birrer, M., Egan, G., Kaye, F., Sausville, E. and Minna, J. The human L-myc gene encodes multiple nuclear phosphoproteins from alternatively processed mRNAs. Mol. Cell. Biol., 8, 4381–4388 (1988).