from those with fatal outcome were selected by means of signal-to-noise metrics (Golub *et al.*, 1999). Briefly, when prognosis-favorable and fatal patients are defined as class 0 and 1, signal-to-noise statistic (S_x) is calculated as $S_x=(\mu_{class0}-\mu_{class1}/\sigma_{class0}+\sigma_{class1})$, where, for each gene, μ_{class0} represents the mean value and σ_{class0} represents the standard deviation for that gene in all samples of class 0. We selected top-ranked genes based on the absolute values for S_x of each gene. A weighted-voting classification algorithm was employed to predict outcome by using the genes selected as described above and the resulting outcome classifiers were tested by means of 'leave-one-out' cross-validation (Golub *et al.*, 1999). In this scheme, the algorithm can also be used to find the decision boundaries between the class means: $b_x=(\mu_{class0}+\mu_{class1})/2$ for each gene in addition to computing S_x. To predict the class of a test sample γ, each gene x in the predictive gene set has a vote based on the expression in this sample (g_x^γ) and b_x: $V_x=S_x\ (g_x^\gamma-b_x)$ and the final vote for class 0 or 1 is *sign* $(\sum_x V_x)$. The votes were summed to determine the winning class, as well as a 'prediction strength' (PS), which is a measure of the margin of victory that ranges from 0 to 1. The PS is defined as $PS=(V_{win}-V_{lose})/(V_{win}+V_{lose})$, where V_{win} and V_{lose} are the total votes for the winning and losing classes. The measure PS reflects the relative margin of victory of the vote.

Permutation tests

For the permutation tests of statistical significance of class-specific markers, 10 000 random permutations of the sample labels (dead or alive) were performed with the dataset, and the signal-to-noise ratio was recalculated for each gene and for each class label permutation. Each gene was thus assigned a *P*-value based upon the frequency by which the permutated signal-to-noise statistics were better than the actual signal-to-noise ratio.

A total of 10 000 random sets of genes of the same numbers used for the actual outcome classifiers were generated from the pool of 900 informative genes, and each of these permuted gene sets was then used for prediction with an independent weighted-voting algorithm by using the leave-one-out cross validation strategy. The *P*-values for each outcome classifier were assigned based on the frequency (N) with which random models performed better than models generated by using the significant genes selected by signal-to-noise, or $P=N/10000$. The probability that outcome classifiers constructed by chance might outperform the actual classifiers was also examined. In this test, the outcome labels were randomly permutated 10 000 times and for each instance, a set of models was built by using the same set of parameters, for example, the number of predictive genes, as the ones used for finding the actual outcome classifiers. The probabilities of chance construction were calculated based on the frequencies with which random models performed better than the actual models in leave-one-out cross-validation as well as in further validation with independent datasets.

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Advances in Brief

Reduced Expression of the *let-7* MicroRNAs in Human Lung Cancers in Association with Shortened Postoperative Survival

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Abstract

In this study, we report for the first time reduced expression of the *let-7* microRNA in human lung cancers. Interestingly, 143 lung cancer cases that had undergone potentially curative resection could be classified into two major groups according to *let-7* expression in unsupervised hierarchical analysis, showing significantly shorter survival after potentially curative resection in cases with reduced *let-7* expression ($P = 0.0003$). Multivariate COX regression analysis showed this prognostic impact to be independent of disease stage (hazard ratio = 2.17; $P = 0.009$). In addition, overexpression of *let-7* in A549 lung adenocarcinoma cell line inhibited lung cancer cell growth *in vitro*. This study represents the first report of reduced expression of *let-7* and the potential clinical and biological effects of such a microRNA alteration.

Introduction

Cells contain a variety of noncoding RNAs, which perform a multitude of functions. Recently, microRNAs (miRNAs), an abundant class of small noncoding RNAs of about 22 nucleotides in length, have been recognized as being numerous and phylogenetically well conserved (1). The miRNA species are encoded by genes that are presumably transcribed into single or clustered miRNA precursors, which are converted to mature forms of miRNAs through stepwise processing including generation of ~70 nucleotide pre-miRNA with a characteristic hairpin structure from the longer nascent transcripts (pri-miRNA) and the following Dicer-mediated processing into mature forms (2–5). Although thus far over 300 miRNA genes have been discovered in various organisms (6–10), including humans, their precise physiological functions are largely unknown except for a handful of miRNAs (11–17), and their potential pathological involvement including oncogenesis is yet to be explored.

The *Caenorhabditis elegans let-7*miRNA is to date the best-studied example along with *lin-4* of the same worm (11–15), both of which were initially identified by genetic analysis of the developmental timing defects of mutants. The *let-7* miRNA, which starts to be expressed during the late developmental stage, acts as a post-transcriptional repressor of *lin-41, hbl-1/lin-57* and perhaps other genes that contain sequences imprecisely complementary to the miRNA in their 3′ untranslated regions. The expression levels of the human *let-7* gene have been shown to vary among various adult tissues, lung being one of the tissues with most abundant expression of *let-7* (18).

In this study, we show for the first time that expression levels of *let-7* are frequently reduced in lung cancers both *in vitro* and *in vivo*. Furthermore, lung cancer patients with reduced *let-7* expression were found to have significantly worse prognosis after potentially curative resection, and this prognostic impact of reduced *let-7* expression appears to be independent of disease stage in multivariate COX regression analysis. In addition, we show that overexpression of *let-7* inhibits growth of lung cancer cells *in vitro*.

Materials and Methods

Study Population. This study dealt with 159 nonsmall cell lung carcinoma (NSCLC) tissue specimens collected with the approval of the institutional review board of the Aichi Cancer Center. The specimens from 143 cases (105 adenocarcinomas, 25 squamous cell carcinomas, 9 large cell carcinomas, and 4 adenosquamous cell carcinomas), which had been followed up for >5 years after potentially curative resection, were used specifically for studying the prognostic significance of *let-7*. These 143 cases consisted of 90 female and 53 male patients with a median age of 62 (range, 32–84), and with 75 in stage I, 19 in stage II, and 49 in stage III.

Preparation of Cell Line and Tissue Samples. All of the human NSCLC cell lines analyzed were cultured with 5% (v/v) FCS-containing RPMI 1640 at 37°C with 5% CO_2. BEAS-2B and HPL1D (19) cells were cultured with 1% (v/v) FCS-containing Ham's F-12 supplemented with bovine insulin (5 μg/ml), human transferrin (5 μg/ml), 10^{-7} M hydrocortisone, 2×10^{-10} M triiode thyronine, penicillin (100 IU/ml), and streptomycin (100 μg/ml) at 37°C with 5% CO_2. The tumor specimens were homogenized in guanidine isothiocyanate homogenization buffer immediately after resection and stored at −30°C until use with the approval of the institutional review board. Processing of all cell lines and tissue samples for RNA extraction were performed according to the standard procedures.

Northern Blotting. Ten μg of RNA were separated on a 15% denaturing polyacrylamide gel. The RNA was then transferred to Zeta-Probe GT Blotting Membranes electrophoretically overnight. Probes (let-7; 5′-TACTATACAACCTACTACCTCAATTTGCC and 5S; 5′-TTAGCTTCCGAGATCAGACGA) were generated by T4 polynucleotide kinase (New England Biolabs, Beverly, MA) mediated end-labeling of DNA oligonucleotides with [γ-^{32}P]ATP. Prehybridization and hybridization were carried out using hybridization buffer (0.25 M sodium phosphate (pH 7.2), 7% SDS, 0.5% sodium PP_i). The most stringent wash was carried out in 2× SSC and 1% SDS at 37.5°C.

Real-Time Reverse Transcription-PCR. Real-time reverse transcription-PCR was performed using an ABI Prism 7900 Sequence Detection System (Perkin-Elmer Applied Biosystems, Foster City, CA), the SYBR Green PCR Master Mix (Perkin-Elmer Applied Biosystems), and random-primed cDNAs (corresponding to 20 ng of total RNA extracted from tissue samples).

The primer pairs used were *let-7a-1*S (sense; 5′-CCTGGATGTTCTCTTCACTG) and *let-7a-1*AS (antisense; 5′-GCCTGGATGCAGACTTTTCT); *let-7a-2*S (sense; 5′-TTCCAGCCATTGTGACTGCA) and *let-7a-2*AS (antisense; 5′-CTCACCATGTTGTTTAGTGC); *let-7a-3*S (sense; 5′-ACCAAGACCGACTGCCCTTT) and *let-7a-3*AS (antisense; 5′-CTCTGTCCACCGCAGATATT); *let-7f-1*S (sense; 5′-TGTACTTTCCATTCCAGAAG) and *let-7f-1*AS (antisense; 5′-TAATGCAGCAAGTCTACTCC); *let-7f-2*S (sense; 5′-TGAAGATGGACACTGGTGCT) and *let-7f-2*AS (antisense; 5′-

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Note: J. Takamizawa and H. Konishi contributed equally to the present study. H. Konishi is currently at the Sidney Kimmel Comprehensive Cancer Center, Johns Hopkins University, Baltimore, MD.

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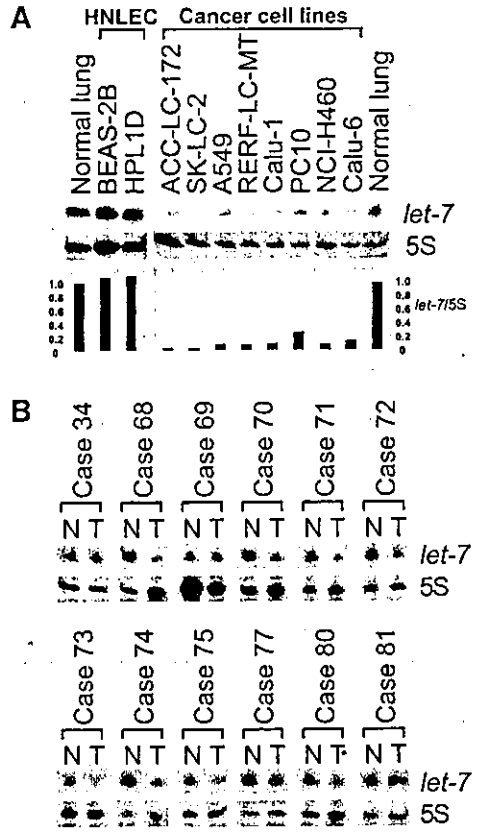

Fig. 1. Northern blot analysis of *let-7* expression in primary lung cancers. *A*, representative Northern blot analysis in lung cancer cell lines *in vitro*. BEAS-2B and HPL1D, immortalized human normal bronchial and peripheral lung epithelial cell lines, respectively. HNLEC, human normal lung epithelial cell lines. *B*, representative Northern blot analysis of primary lung cancer specimens *in vivo*. 5S rRNA served as a loading control. N, normal lung; T, lung cancer.

CAGTCGGAGAAGAAGTGTAC); and 5S–S (sense; 5′-TACGGCCATAC-CACCCTGAA) and 5S-AS (antisense; 5′-TAACCAGGCCCGACCCTGCT). To quantify the expression level of the *let-7* genes, standard curves were made using serially diluted pBluescriptIISK (−) inserted with each PCR product into the *Eco*RV site. PCR amplification consisted of 55 cycles (95°C for 30 s, 56°C to 60°C optimized for each primer set for 30 s and 72°C for 15 s) after the initial denaturation step (95°C for 10 min). Expression levels of the *let-7* genes were based on the amount of the target message relative to the 5S rRNA control, to normalize the initial input of total RNA.

Hierarchical Clustering. We used the Eisen CLUSTER and TREEVIEW programs for hierarchical clustering and visualization of data sets. Before applying the clustering algorithm, we log-transformed the fluorescence ratio for each expression and then average centered the data for all samples. Agglomerative hierarchical clustering was applied using the complete linkage method to investigate whether there was evidence for natural groupings of tumor samples based on correlations between gene-expression profiles.

Statistical Analysis. The Kaplan-Meier method was used to estimate survival as a function of time, and survival differences were analyzed by the log-rank test. Cox regression analysis of factors potentially related to survival was performed to identify which independent factors might jointly have a significant influence on survival.

Colony Formation Assay. The *let-7* expression construct and a control plasmid were constructed by the cloning of annealed oligonucleotides of *let-7a* (sense, 5′-GATCCCCTGAGGTAGTAGGTTGTATAGTTTTT and antisense, 5′-AGCTAAAAACTATACAACCTACTACCTCAGGG), *let-7f* (sense, 5′-GATCCCCTGAGGTAGTAGATTGTATAGTTTTT and antisense, 5′-AGCTA-AAAACTATACAATCTACTACCTCAGGG), or control (sense, 5′-GATC-CCCTTTTTTTTGGAAA and antisense, 5′-AGCTTTTCCAAAAAAAAGGG) into pH1-RNApuro, in which expression of a gene is under the control of the RNA polymerase III H1-RNA gene promoter prepared by PCR amplification of human genomic DNA. The *let-7a* and *-7f* expression constructs were transfected into A549 lung adenocarcinoma cell line using the FuGENE 6 reagent (Roche, Inc. Basel, Switzerland) according to the manufacturer's instructions. Cells were selected by the addition of puromycin (2 μg/ml) 3 days after the transfection and cultured at 37°C for 2 weeks. After 2 weeks of puromycin selection, the plates were stained with Giemsa and scored for the number of resistant colonies.

Results

Reduced Expression of *let-7* in Human Lung Cancers in Both *in Vitro* and *in Vivo*. Northern blot analysis was first performed to analyze *let-7* expression in 20 human lung cancer cell lines as well as in two immortalized human normal lung epithelial cell lines (Fig. 1*A*). The mature form of *let-7* miRNA was readily detectable in both immortalized lung epithelial cell lines at a level comparable with that in normal lung tissues. In marked contrast, a significant reduction (>80%) in the expression levels of *let-7* was observed in 60% (12 of 20) of lung cancer cell lines. Expression levels of *let-7* in primary human lung cancer tissues taken directly from surgically treated patients, in which sufficient RNA were available, were further analyzed by Northern blot analysis. Consequently, 44% (7

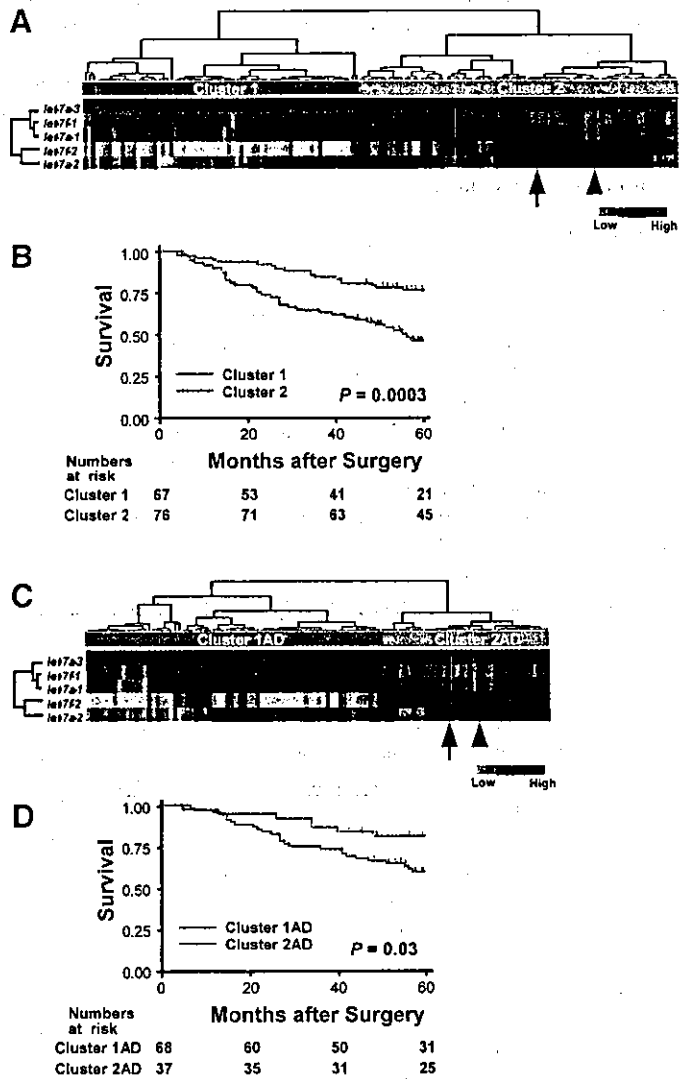

Fig. 2. Hierarchical clustering and Kaplan-Meier survival curves based on expression of *let-7* microRNA (miRNA) isoforms. *A*, results of unsupervised hierarchical clustering of the entire cohort of 143 nonsmall cell lung carcinoma (NSCLC) cases. *B*, Kaplan-Meier survival curves for NSCLC patients who were classified into clusters 1 and 2. The difference in postoperative survival between clusters 1 and 2 was highly significant ($P = 0.0003$ by log-rank test). *C*, results of unsupervised hierarchical clustering of the 105 adenocarcinoma cases. *D*, Kaplan-Meier survival curves for adenocarcinoma cases belonging to either cluster 1AD or 2AD. The difference in postoperative survival between clusters 1AD and 2AD was also statistically significant ($P = 0.03$ by log-rank test). *Arrows* and *arrow heads*, mixture of RNAs of 38 and 120 normal human peripheral lung tissues, respectively.

of 16) of the cases examined were found to exhibit >80% reduction in *let-7* expression when compared with that in the corresponding normal lung tissues (Fig. 1*B*). A more frequent occurrence of reduced *let-7* expression in cell lines *in vitro* may be related to the inevitable contamination of normal stromal/inflammatory cells in tumor tissues *in vivo* or, alternatively, this may reflect *in vitro* selection of cells with reduced *let-7* in the process of the establishment of cell lines. These findings thus clearly showed the frequent occurrence of a significant reduction in *let-7* miRNA expression in lung cancers.

Prognostic Impact of Reduced *let-7* Expression in Surgically Treated Lung Cancer Patients. We next wished to investigate whether reduced *let-7* expression has any relation to clinicopathological characteristics of lung cancers in an isoform-specific manner. To this end, 143 lung cancer cases, which had undergone potential curative resection of NSCLCs, were examined by real-time reverse transcription-PCR analysis using *let-7* isoforms-specific oligonucleotide primers. Expression levels of *let-7* pri-miRNAs were consequently shown to vary significantly among lung cancer cases, although they tended to be coordinately regulated. The most abundant species were *let-7a-1* and *let-7f-1*, which are known to be clustered within a few hundred bases in the human genome (6) and could be amplified together by reverse transcription-PCR (data not shown). We used unsupervised hierarchical clustering to classify the 143 resected human NSCLC cases in an unbiased manner without using any information on the identity of the samples. This procedure resulted in the classification of NSCLC cases into two major classes based on similarities in *let-7* expression (Fig. 2*A*). Except for a significant association between cluster 1 with low *let-7* expression and higher disease stages ($P = 0.004$ by the λ^2 test), no other significant associations were found between the clusters and various clinicopathological features including age, sex, histology, primary tumor status (pT), and differentiation grade. Of special interest was a striking difference in the postoperative survival of patients between the two clusters. The Kaplan-Meier survival curves demonstrated that patients belonging to cluster 1 were at a significantly greater risk of an earlier death than those classified as cluster 2 ($P = 0.0003$ by the log-rank test; Fig. 2*B*). A separate study analyzed the prognostic significance of *let-7* in adenocarcinomas, which constitute the major proportion of lung cancers in Japan as well as in other countries such as the United States. We found that adenocarcinoma cases can also be divided into two major clusters, again showing that patients in cluster 1AD with low *let-7* expression had significantly shorter survival than those in cluster 2AD with high *let-7* expression ($P = 0.03$ by the log-rank test; Fig. 2, C and D).

Univariate Cox regression analysis was then performed for the entire cohort and showed that, in addition to disease stage ($P < 0.001$; Table 1), classification into cluster 1 with characteristically low *let-7* expression is a significant predictive factor for poor prognosis ($P < 0.001$). Cox proportional hazards modeling was then conducted to identify which independent factors would jointly have a significant influence on survival (Table 1). The inter-relationship of possible prognostic factors and survival was analyzed, using age, sex, histological type, smoking history, disease stage, and the *let-7*-defined cluster as variables, resulting in the identification of *let-7*-defined cluster as a significant, independent prognostic factor in surgically treated NSCLC patients after potentially curative resection ($P = 0.009$) in addition to disease stage ($P < 0.001$). The hazard ratio of earlier death was 2.17 (95% confidence interval, 1.21–3.89) for clusters 1 *versus* 2 and 3.49 (95% confidence interval, 1.89–6.42) for pathological stage II/III *versus* pathological stage I. Taken together, expression levels of *let-7* seemed to have a significant impact on the postoperative survival of NSCLC patients.

Table 1 Cox regression analysis of various prognostic factors for postoperative survival of lung cancer patients

Variables	Hazard ratio (95% CI[a])	Unfavorable/favorable	*P*
Univariate analysis			
Age (yr)	1.70 (0.97–2.99)	≥62/<62	0.063
Sex	1.34 (0.75–2.38)	Male/female	0.323
Histology	1.30 (0.67–2.52)	Squamous/non-squamous	0.443
Smoking history	1.42 (0.80–2.51)	Smoker/non-smoker	0.233
Disease stage	3.89 (2.14–7.08)	II-III/I	<0.001
let-7	2.78 (1.56–4.89)	Cluster 1/cluster 2	<0.001
Multivariate analysis			
Age (yr)	1.68 (0.95–2.97)	≥62/<62	0.076
Sex	1.18 (0.44–3.13)	Male/female	0.741
Histology	1.03 (0.49–2.16)	Non-squamous/squamous	0.942
Smoking history	1.07 (0.41–2.82)	Non-smoker/smoker	0.889
Disease stage	3.49 (1.89–6.42)	II- III/I	<0.001
let-7	2.17 (1.21–3.89)	Cluster 1/cluster 2	0.009

[a] 95% CI, 95% confidence interval.

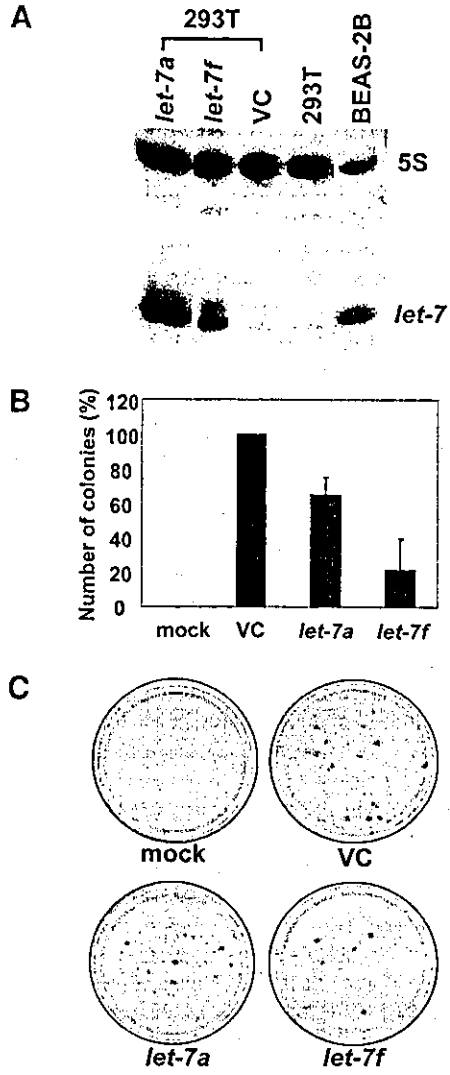

Fig. 3. Introduction of *let-7* into A549 lung adenocarcinoma cell line. *A*, results of Northern blot analysis confirming expression of *let-7a* and *let-7f* isoforms. *B*, graphic presentation of a representative colony formation assay by the introduction of exogenous *let-7*. Similar results were obtained in five independent assays done in triplicate. *C*, representative dishes showing reduced colony formation by overexpression of exogenously introduced *let-7*.

Growth Suppression of Lung Cancer Cells by Overexpression of Exogenous *let-7*. The identification of a reduced expression of *let-7* in lung cancers, in association with a shortened survival, prompted us to explore the possible biological significance of *let-7* in lung cancer development. As an initial step, we introduced *let-7* into a lung cancer cell line by using expression constructs, which were designed to synthesize mature miRNAs of two predominant *let-7* isoforms, *let-7a* and *let-7f*, under the control of the RNA polymerase-III H1-RNA gene promoter. We confirmed that these expression constructs could work as expected using 293T cells (Fig. 3*A*). Overexpression of *let-7f* in A549 lung adenocarcinoma cell line resulted in a 78.6% reduction in the number of colonies, whereas the introduction of *let-7a* also showed similar but a more modest growth-inhibitory effect (Figs. 3, *B* and *C*). Similar results were obtained in five independent experiments, which were done in triplicate using three independent preparations of plasmid DNAs.

Discussion

It has become apparent that genomic information for transcribing miRNAs is indeed implemented in the human genome (6, 9), but extremely little information is available regarding their physiological

and pathological roles. This is the first demonstration that expression levels of *let-7* miRNA, which to date is one of the best-studied miRNAs, are altered in human lung cancers. Furthermore, we have shown that reduced *let-7* expression is significantly associated with shortened postoperative survival and that overexpression of *let-7* results in the inhibition of lung cancer cell growth. Altogether, these findings suggest that reduced expression of *let-7* may play a role in the pathogenesis of lung cancers.

Very little information is available at the moment with regard to the potential pathological roles of miRNAs. Two proteins (Gemin 3 and Gemin 4), which are components of the protein complex related to spinal muscular atrophy, are also known to be components of a ribonucleoprotein complex containing miRNAs (microRNP; Ref. 9), whereas the *Drosophila* homologue of fragile X mental retardation protein has been shown to be a component of RNA-induced silencing complex/microRNPs (20, 21). This circumstantial evidence suggests the possibility of the involvement of miRNA machineries in these diseases. As for links between cancer and miRNA, Calin *et al.* (22) reported frequent down-regulation of *miR15* and *miR16* in chronic lymphocytic leukemia, whereas Michael *et al.* (23) recently reported reduced expression of *miR-143* and *miR-145* in human colon cancers. In contrast to these studies, which did not address the question of whether reduced expression of miRNAs has any influence on clinicopathological features, this study clearly shows that reduced *let-7* expression is indeed significantly associated with the shortened survival of patients. Because no changes in *let-7* expression were reported in colon cancers (23), it is possible that miRNAs may be distinctly involved in the pathogenesis of these two most common cancers of adults and possibly in other types of human cancers.

It has been shown that the *let-7* gene regulates developmental timing in *C. elegans* and that mutant worms lacking *let-7* fail to properly execute a larval-to-adult switch in hypodermal cell development (13). Although *lin-41* is known to be post-transcriptionally repressed by *let-7* (24), it is not inconceivable that other genes may also be targeted by *let-7*, because of the requirement of imprecise base-pairing for miRNA-mediated translational repression (1). Indeed, *hbl-1/lin-57* was recently reported to be targeted by *let-7* (14, 15), whereas a few additional genes have also been predicted to be a potential target for *let-7* (24, 25). Interestingly, such potential targets include *LIM kinase 2* (25), which belongs to a gene family having a role in the regulation of cell shape and motility as well as possibly in metastasis. One could speculate that the change in miRNA expression as is seen in this study might be an efficient strategy for cancer cells to simultaneously alter the expression profile of a series of genes. Alterations in miRNA expression may accordingly confer cancer cells with selective growth advantage, allowing them to form a distant metastasis and resulting in the consequential death of the patient. This scheme may be consistent with the present finding of the significant prognostic impact of *let-7* expression. One might argue that reduced expression of *let-7* in lung cancers may merely reflect its oncofetal regulation, because fetal lung exhibited considerably lower *let-7* expression than adult lung (data not shown). However, growth-inhibitory effects of overexpressed *let-7* in A549 adenocarcinoma cell line argue against this possibility. Taken together, these findings suggest the potential involvement of reduction in *let-7* expression in the pathogenesis of this fatal disease, although the results obtained with overexpression of mature miRNA need to be interpreted cautiously and await further experimental clarification.

In this study, we observed that various *let-7* pri-miRNA isoforms were coordinately regulated, *let-7a-1* and *let-7f-1* being the most predominant. In this connection, it should be noted that some of the *let-7* pri-miRNAs give rise to identical mature miRNA isoforms, and the others may also have presumably very similar, if not identical, spectra of the target genes (6). It is uncertain at the moment how the expression levels of various *let-7* isoforms are coordinated, and this remains an intriguing question awaiting further investigation.

In conclusion, we have shown for the first time that *let-7* expression is frequently reduced in lung cancers and that alterations in the miRNA expression may have a prognostic impact on the survival of surgically treated lung cancer patients. These findings warrant additional studies to investigate whether *let-7* alterations are also involved in other types of human cancers and how altered miRNA expression would manifest the biological and biochemical consequences in the development of human cancers. Accordingly, future identification of the downstream targets for *let-7* may provide clues to develop a novel therapeutic means. It is envisaged that such future studies may ultimately provide a foundation for a new paradigm of the involvement of noncoding small RNA species, miRNA, in human oncogenesis.

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Identification of an epitope from the epithelial cell adhesion molecule eliciting HLA-A*2402-restricted cytotoxic T-lymphocyte responses

Key words:
autoimmunity; CTL; dendritic cells; epithelial cell adhesion molecule; HLA-A24; immunotherapy

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Abstract: Because the epithelial cell adhesion molecule (Ep-CAM) is expressed in almost all carcinomas and human leucocyte antigen (HLA)-A*2402 is the most common allele in many ethnic groups, including Japanese, the identification of peptide sequences, which elicit HLA-A*2402-restricted Ep-CAM-specific cytotoxic T-lymphocyte (CTL) responses, would facilitate specific immunotherapy for various histological types of carcinomas. An epitope was identified through the following steps: (i) computer-based epitope prediction from the amino acid sequence of Ep-CAM, (ii) major histocompatibility complex (MHC) stabilization assay to determine the affinity of the predicted peptide with HLA-A*2402 molecules, (iii) stimulation of $CD8^+$ T cells with peptide-pulsed dendritic cells and (iv) testing the CTL specificity by means of enzyme-linked immunospot (ELISPOT) assays, CTL assays and MHC/peptide-tetramer staining. Peripheral $CD8^+$ T cells of four of five healthy donors after three rounds of stimulation with the peptide Ep-$CAM_{173-181}$ (RYQLDPKFI) secreted interferon-γ in ELISPOT assays when exposed to the peptide. A CTL clone specific to the peptide efficiently lysed Ep-CAM-expressing cancer cell lines in an HLA-A*2402-restricted fashion. Endogenous processing and presentation of the peptide in a lung cancer cell line were confirmed by means of cold target inhibition assays. The CTL clone was also lytic to normal bronchial epithelial cells but to a lesser extent at low effector: target ratios. All these data suggest that the peptide-specific CTL responses may play some roles both in anti-cancer and autoimmune reactions. The peptide should prove useful to study anti-Ep-CAM CTL responses among population possessing HLA-A*2402.

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Cytotoxic T-lymphocytes (CTLs) have become widely accepted as important players in resistance to cancer. Although various CTL epitopes of tumour-associated antigens have been identified so far (1, 2), the search for additional epitopes continues, because the expression of tumour antigens is heterogeneous among tumours of various histological origins, various patients and between individual lesions. From the clinical point of view, molecular characterization of

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additional tumour antigens is crucial for successful immunotherapy, because immunoselection of antigen-negative tumour cell variants has been observed during peptide vaccination (3–5).

Epithelial cell adhesion molecule (Ep-CAM), also referred to as EGP-2, 17-1A, GA733-2, KSA or PE-35 (6–10), was originally reported as a serologically defined surface antigen, highly expressed on many carcinomas of diverse histological origins, such as colon (11), lung (12), head and neck (13) and breast tumours (14), but with limited expression by normal epithelial cells (15, 16). Its function is to mediate Ca^{2+}-independent homotypic cell–cell adhesion. Because of its intensive and uniform expression in a variety of human tumours, Ep-CAM has become one of the most attractive targets for immunotherapy with monoclonal antibodies, or even for gene therapy (17). Treatment of a series of patients suffering from Dukes' C colorectal carcinoma with a monoclonal antibody against Ep-CAM, namely 17-1A, has been found to reduce mortality and recurrence (18, 19). Recently, it was reported that HLA-A*0201-restricted Ep-CAM-derived peptide-specific CTLs can lyse epithelial tumour cells but not normal cells (20, 21). Immunotherapy using such epitope peptides has potential efficacy.

Using a bioinformatic approach, in the present study, we first predicted seven peptide sequences in Ep-CAM, which might bind to HLA-A*2402 molecules, the most common allele in Japanese (more than 60%) and also present in persons of European descent (nearly 20%). Specific CTL was successfully induced in four of five healthy donors by using Ep-$CAM_{173-181}$ (RYQLDPKFI) and a $CD8^+$ CTL clone specific to this peptide showed cytotoxicity against HLA-$A24^+$ Ep-CAM^+ but not HLA-$A24^-$ cancer cells. Cold target inhibition assays suggested that the peptide was naturally processed and was presented on the surfaces of HLA-$A24^+$ Ep-CAM^+ cancer cells. The fine specificity of the peptide-specific CTL was extensively studied and the results were discussed in the light of anti-cancer and anti-self cellular immunity.

Materials and methods

Donors and cell lines

The study design and purpose, which had been approved by the Institutional Review Board of Aichi Cancer Center, Nagoya, Japan, were explained fully to all donors. Peripheral blood was obtained from five HLA-A24-positive healthy donors and peripheral blood mononuclear cells (PBMCs) were isolated by means of centrifugation on a Ficoll-Paque (Pharmacia, Piscataway, NJ) density gradient.

Human cancer cell lines – LU99, HSC-2, MKN28, MKN45 and COLO320DM cells – were purchased from the Japanese Collection of Research Bioresources (Tokyo, Japan) and LC-1/sq from RIKEN Cell Bank (Tsukuba, Japan). LC-1/sq cells were maintained in 45% RPMI 1640 medium (Sigma, St Louis, MO) and 45% Ham's F12 medium (Sigma) supplemented with 10% fetal calf serum (FCS) (Life Technologies Limited, Auckland, New Zealand), L-glutamine, penicillin and streptomycin. COLO320DM and MKN28 were maintained in Dulbecco's modified Eagle medium (Sigma) with the same supplements. The other cancer cell lines were cultured in RPMI1640 medium with the same supplements (referred to as complete medium). HLA-A24-positive, normal human bronchial epithelial cells, designated as NHBE, were cultured according to the manufacturer's recommendations (CC2540, Clonetics Corp, BioWhittaker, Walkersville, MD). The HLA-A*2402 transfectants – T2-A24, QG56-A24 and A549-A24 – were established and were cultured as previously described (22, 23).

Reverse transcription polymerase chain reaction

Using a GenElute mRNA Miniprep kit (Sigma Chemical Co., St Louis, MO), total RNA was extracted from cultured cell lines. Gene-specific oligonucleotide primers were synthesized at Proligo (Kyoto, Japan) and were used in order to evaluate the mRNA expression of Ep-CAM. Forward and reverse primers used were as follows: ATG GCG CCC CCG CAG GTC CT and TTA TGC ATT GAG TTC CCT ATG CAT CTC ACC. Reverse transcription polymerase chain reaction (RT-PCR) was performed by using a thermal cycler (Perkin-Elmer, Wellesley, MA) and products were analysed by means of 1.5% agarose gel electrophoresis with ethidium bromide visualization.

Western blot analysis

Western blot analysis was performed as described previously (24) with slight modifications. Briefly, aliquots of 130-μg protein from the post-nuclear supernatant of the cell lysate were applied to 12% SDS-PAGE and were blotted onto Immobilon-P membranes (Millipore Corporation, Bedford, MA). After probing with a monoclonal antibody specific to Ep-CAM (clone 323/A3, Laboratory Vision, Fremont, CA), followed by peroxidase-conjugated goat anti-mouse immunoglobulin G (IgG) (Zymed, San Francisco, CA), proteins were visualized with the help of an ECL Western blot detection system (Amersham Biosciences, Buckinghamshire, UK).

Synthetic peptides

In order to identify potential HLA-A24-binding peptides within Ep-CAM (accession number M33011), we employed a computer-based program accessed through the World Wide Web site BioInformatics & Molecular Analysis Section (BIMAS) HLA peptide-binding predictions (available at http://bimas.dcrt.nih.gov/molbio/hla_bind/). Most peptides were synthesized with a Cleaved PepSet from Mimotope

(Melbourne, Australia), dissolved in 100 µl dimethyl sulfoxide and further diluted in 40% acetonitrile, 0.1 M HEPES (pH 7.4), where necessary. Characteristics of the seven synthetic peptides, designated as Ep_{31}, Ep_{173}, Ep_{185}, Ep_{250}, Ep_{225}, Ep_{296} and Ep_{304}, have been listed in Table 1. A human immunodeficiency virus-1 (HIV-1) envelope peptide RYLRDQQLL (25) (residues 584–592, designated as ENV_{584}) and an EBV (Epstein-Barr virus) latent membrane protein 2 peptide TYGPVFMCL (26) (residues 419–427, EBV-LMP2419) were synthesized by Toray Research Center (Kamakura, Japan).

Cell staining and flow cytometric analysis

Surface expression of HLA-A24 and Ep-CAM molecules was examined with the help of indirect immunofluorescence by using an anti-HLA-A24 monoclonal antibody (One Lambda, Inc., Canoga Park, CA), the anti-Ep-CAM monoclonal antibody and FITC-labelled (fluorescein isothiocyanate) anti-mouse IgG $F(ab')_2$ fragments (IMMUNOTECH, Marseille, France). MHC/peptide tetramers were produced as previously described (22, 27). The Ep-CAM-specific $CD8^+$ T cells were stained with PE-labelled HLA-A*2402 tetramers incorporating the Ep-CAM peptide, Ep_{173} (designated as the HLA-A24/Ep_{173} tetramer) or the HIV-1 peptide, ENV_{584} (HLA-A24/ENV_{584} tetramer). Flow cytometric analysis of the stained cells was performed by means of a FACSCalibur (Becton Dickinson, San Jose, CA) and the data were analysed with the help of CellQuest software (Becton Dickinson).

MHC stabilization assay

The seven synthesized peptides were used in an MHC stabilization assay by using T2-A24 cells as described earlier (22). Briefly, T2-A24 cells (2×10^5) were incubated with 200 µl of RPMI1640 containing 0.1% FCS and 5×10^{-5} M β-mercaptoethanol and each of the peptides at a concentration of 10 µM at 26°C for 16 h, followed by incubation at 37°C for 3 h. Surface HLA-A24 molecules were then stained with the anti-A24 monoclonal antibody and FITC-labelled anti-mouse IgG. Expression was measured in the FACSCalibur, and mean fluorescence intensity (MFI) was recorded. The percentage of MFI increase was calculated as follows: percentage of MFI increase = 100 × (MFI with the given peptide − MFI without the peptide)/(MFI without the peptide).

Characteristics of epithelial cell adhesion molecule (Ep-CAM) candidate peptides

Peptide designation	Amino acid sequence	Position	Sequence length	Score[a]	Percentage of MFI increase[b]
Ep_{31}	NYKLAVNCF	31–39	9	120	85
Ep_{173}	RYQLDPKFI	173–181	9	150	102
Ep_{185}	LYENNVITI	185–193	9	75	79
Ep_{225}	LFHSKKMDL	225–233	9	20	29
Ep_{250}	YYVDEKAPEF	250–259	10	198	57
Ep_{296}	KYEKAEIKEM	296–305	10	83	24
Ep_{304}	EMGEMHREL	304–312	9	5	16

[a]Estimated half-time of dissociation from HLA-A24 molecules (min), obtained with a computer program (World Wide Web site BioInformatics & Molecular Analysis Section (BIMAS) HLA peptide-binding predictions).
[b]Synthetic peptides were tested for binding to human HLA-A*2402 molecules in MHC stabilization assays as described in the section entitled 'Materials and methods.' MFI, mean fluorescence intensity.

Table 1

Generation of Ep-CAM peptide-specific CTL lines and clones

Peripheral blood monocyte-derived dendritic cells (DCs) were generated as described previously (28). Briefly, plastic adherent cells were isolated from PBMCs and were cultured in RPMI1640 medium supplemented with 5% heat-inactivated human serum, 10 ng/ml of recombinant human interleukin-4 (IL-4) (R&D Systems, Minneapolis, MN) and 50 ng/ml of recombinant human granulocyte-macrophage colony-stimulating factor (R&D Systems). On day 1 of incubation, 10 ng/ml of IL-1β PeproTech, Rocky Hill, NJ), 50 ng/ml of recombinant human tumour necrosis factor-α (TNF-α) (PeproTech) and 1 µM prostaglandin E_2 (Cayman Chemical Company, Ann Arbor, MI) was added for maturation. On days 2 or 3, the cells were harvested and were confirmed to express mature DC-associated antigens, such as CD1a, CD80, CD83, CD86 and HLA class-II molecules (data not shown). The DCs were pulsed with each of the synthetic peptides at a concentration of 10 µM in AIM-V medium (Gibco, Grand Island, NY) supplemented with 5×10^{-5} M β-mercaptoethanol for 2–4 h at room temperature and were irradiated (33 Gy). Thereafter, the DCs (1×10^5) were co-cultured with autologous $CD8^+$ T lymphocytes (1×10^6) purified with the aid of CD8 MicroBeads (Miltenyi Biotec, Bergisch Gladbach, Germany) in RPMI1640 medium supplemented with 10% pooled human serum, 25 ng/ml of recombinant human IL-7 (R&D Systems) and 5 ng/ml of recombinant human IL-12 (R&D Systems) in a culture tube. After culture for 7 days, the cells were stimulated again by adding 1×10^5 peptide-pulsed autologous DCs prepared as described above. After culture for 7 additional days, the cells were stimulated a third time in the same manner. One day after each restimulation, recombinant human IL-2 (Takeda Chemical Industries, Osaka, Japan) was added to a final concentration of 20 U/ml. If necessary, rapidly growing cells were split into two to three tubes and were fed with fresh culture medium containing 20 U/ml of IL-2.

In order to establish T-cell clones, limiting dilution of the polyclonal CTLs was performed (22). After 2-week culture in 96-well plates, the specificity of growing cells was examined with CTL–CTL killing assays as previously described (29). Clones that were

killed only when pulsed with the Ep-CAM peptide were transferred into flasks and were expanded as detailed above.

Enzyme-linked immunospot assay

Enzyme-linked immunospot (ELISPOT) assays were performed as previously described (22). A total of 1×10^3 polyclonal $CD8^+$ T cells were co-cultured with peptide-pulsed T2-A24 cells (5×10^4) in wells of the MultiScreen-HA plates (Millipore) coated with 10 μg/ml of anti-interferon-γ (IFN-γ) monoclonal antibody (R&D Systems). All assays were performed in duplicate. After probing with a polyclonal rabbit anti-IFN-γ antibody (Genzyme, Cambridge, MA), followed by exposure to peroxidase-conjugated goat anti-rabbit IgG (Genzyme), IFN-γ spots were visualized and were counted under a dissecting microscope.

CTL assay

Target cells were labelled with ^{51}Cr as previously described (22). In some experiments, pre-determined amounts of blocking antibodies, W6/32 (anti-HLA class-I), MA2.1 (anti-HLA-A2) and A11.1 (anti-HLA-A24) were added to the wells 30 min before adding effector cells in order to determine the HLA restriction. The plates were incubated for 4 h at 37°C, and the supernatants were counted in a γ-counter. The percentage of specific ^{51}Cr release was calculated as follows: 100 × (experimental release − spontaneous release)/(maximum release−spontaneous release).

Cold target inhibition assays

Cold target inhibition assays were performed as described previously (30). Briefly, T2-A24 cells were incubated with the peptide Ep_{173} or EBV-$LMP2_{419}$ at a concentration of 10 μM for 1 h. After extensive washing, the indicated numbers of peptide-loaded cells were incubated with 2×10^4 cytotoxic effector cells for 1 h, and then 2×10^3 ^{51}Cr-labelled PC9 cells were added to each well. Cytotoxicity was assessed as described above.

Results

Selection of potential HLA-A24-binding peptides within Ep-CAM proteins

The computer program was applied in order to identify potential HLA-A*2402-binding peptides within the amino acid sequence of Ep-CAM and to predict HLA-binding peptides, on the basis of estimation of the half-time dissociation of the HLA/peptide complex. The peptide sequences were reviewed according to peptide motifs for HLA-A24 molecules (31–33), and seven peptides were synthesized (Table 1). MHC stabilization assays were performed in order to test their HLA-A*2402-binding efficiency by using T2-A24 cells. Most peptides increased the HLA-A24 expression on the cells, indicating that they bound and stabilized the HLA complexes on the cell surface, but peptide Ep_{304} gave a low value for the percentage of MFI (Table 1) and was excluded from further studies.

Screening of peptides antigenic for anti-Ep-CAM polyclonal CTL lines by means of ELISPOT assay

In order to identify peptides recognized by Ep-CAM-specific CTLs in the context of HLA-A*2402 molecules, $CD8^+$ T cells of five HLA-$A24^+$ healthy donors were stimulated with autologous DCs pulsed with each of the six peptides. After three rounds of stimulation, T cell lines from four donors produced significant numbers of IFN-γ spots when incubated with T2-A24 cells pulsed with peptide Ep_{173} (Fig. 1). Almost no spots were produced with control peptide ENV_{584} (data not shown). After four-time stimulation, the CTL line established from donor 4 specifically stained with the HLA-A24/Ep_{173} tetramer but not the HLA-A24/ENV_{584} tetramer (37.2 *vs* 0.06% of the total $CD8^+$ T cells, Fig. 2A). The intensity of the tetramer-positive cells was homogeneous and stronger than that of tetramer-negative cells by two- to three-fold on a logarithmic scale.

We established a T-cell clone, designated as C27, from limiting dilution culture of the Ep_{173}-specific polyclonal CTL line of donor 4. The study with tetramers indicated that both polyclonal and monoclonal Ep_{173}-specific $CD8^+$ T cells had high-affinity antigen receptors directed to HLA-A*2402/Ep_{173} complexes (Fig. 2B).

While an Ep_{250}-stimulated CTL line from donor 3 also specifically produced IFN-γ spots when incubated with Ep_{250} (Fig. 1), the establishment of Ep_{250}-specific CTL clones was unsuccessful. We, thus, further evaluated the Ep_{173}-specific CTL clone.

Characterization of the peptide Ep_{173}-specific $CD8^+$ CTL clone

Ep-CAM expression of cancer cell lines was examined by means of RT-PCR, Western blot analysis (Fig. 3) and indirect immunofluorescence (Table 2). The data showed concordance of results obtained with the three approaches. Twelve of 15 (80%) cancer cell lines appeared to express Ep-CAM. When HLA-A24 expression was examined with the help of indirect immunofluorescence by using an HLA-A24 monoclonal antibody, 10 were positive (Table 2).

As shown in Fig. 4(A), the Ep_{173}-specific CTL clone, C27, showed cytotoxicity to T2-A24 cells pulsed with Ep_{173} at a peptide

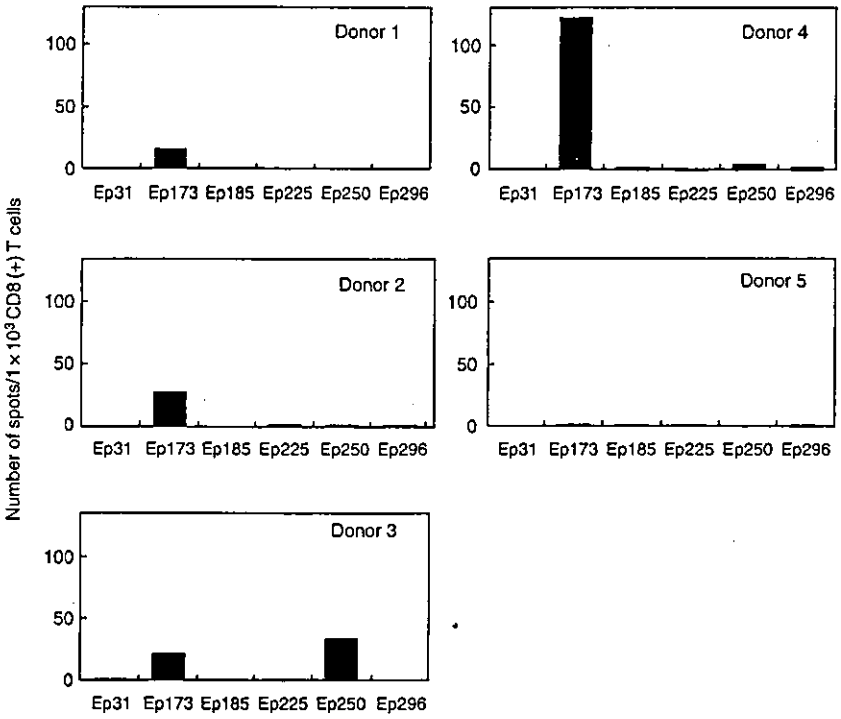

Fig. 1. **Evaluation of CD8⁺ T cell lines by means of enzyme-linked immunospot (ELISPOT) assay.** Aliquots of CD8⁺ T cells (1×10^3) were incubated with T2-A24 cells pulsed with the indicated peptide (10 μM) in wells of the ELISPOT plate. All assays were performed in duplicate.

concentration as low as 100 pM, but not with control peptide EBV-LMP2$_{419}$. Data for cytotoxicity of C27 against various cancer cell lines have been shown in Fig. 5. C27 efficiently lysed lung cancer cell lines PC9, LU99, LC99A and LC-1/sq, an oral squamous cell carcinoma cell line HSC-2 and a gastric cancer cell line MKN45 that expressed both HLA-A24 and Ep-CAM. However, no killing was observed with HLA-A24⁺ Ep-CAM⁻ (11–18, COLO320 DM and A549-A24) or HLA-A24⁻ (either Ep-CAM⁺ or Ep-CAM⁻) cell lines (QG56, A549 and MNK28). After the transfection of the HLA-A*2402 cDNA into HLA-A24⁻ QG56 cells (QG56-A24), they were killed by C27. K562 cells were included in order to assess the degree of NK-like cytotoxicity of C27, which turned out to be negligible.

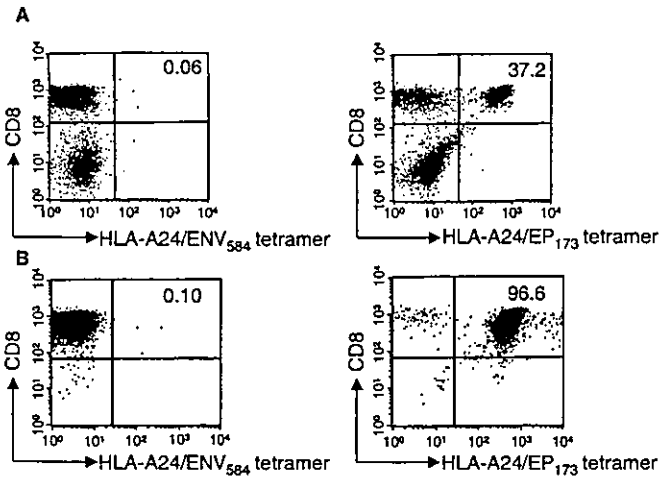

Fig. 2. **Tetramer staining of Ep$_{173}$ peptide-specific CD8⁺ T cells.** Polyclonal CD8⁺ T cells (A) and an Ep$_{173}$-specific cytotoxic T-lymphocyte (CTL) clone – C27 – (B) were stained with HLA-A24 tetramers incorporating Ep$_{173}$ or a control peptide, ENV$_{584}$. The percentages of tetramer-positive cells in CD8⁺ T cells have been shown. HLA, human leucocyte antigen.

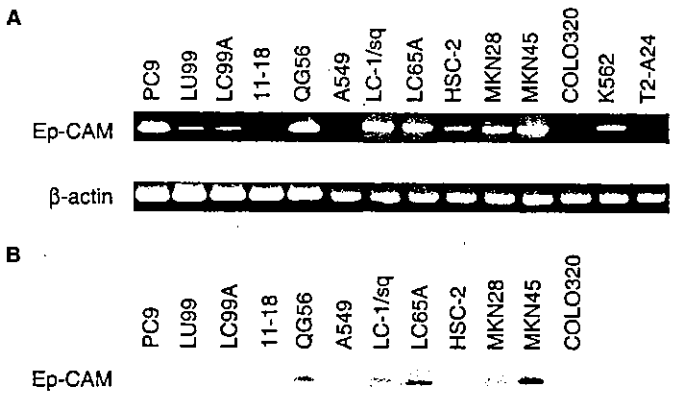

Fig. 3. **Reverse transcription polymerase chain reaction (RT-PCR) and Western blot analysis of epithelial cell adhesion molecule (Ep-CAM) in cancer cell lines.** Semi-quantitative RT-PCR analysis was performed by using specific primers for Ep-CAM and β-actin (A). Western blot analysis of Ep-CAM was performed with a monoclonal antibody specific to Ep-CAM (B).

Characteristics of the cell lines used

Cell line	Origin	Surface expression (MFI[a]) of Ep-CAM	HLA-A24
Lung cancer			
LU99	Giant cell carcinoma	+(22.55)	+(87.16)
PC9	Adenocarcinoma	+(383.67)	+(43.53)
11–18	Adenocarcinoma	−(4.00)	+(73.67)
LC99A	Large cell carcinoma	+(15.32)	+(96.71)
LC65A	Small cell carcinoma	+(307.14)	−(3.67)
LC-1/sq	Squamous cell carcinoma	+(198.94)	+(70.56)
A549	Adenocarcinoma	−(6.40)	−(3.21)
A549-A24	Adenocarcinoma	Not done	+(109.73)
QG56	Squamous cell carcinoma	+(229.13)	−(2.65)
QG56-A24	Squamous cell carcinoma	Not done	+(84.12)
Gastric cancer			
MKN28	Adenocarcinoma	+(722.59)	−(7.67)
MNK45	Adenocarcinoma	+(823.25)	+(47.99)
Colon cancer			
COLO320DM	Adenocarcinoma	−(3.07)	+(35.35)
Others			
HSC-2	Oral squamous cell carcinoma	+(57.15)	+(34.40)
K562	Chronic myelogeneous leukaemia	+(41.45)	−(5.23)
T2-A24	B × T hybrid cells	−(3.05)	+(197.75)
NHBE	Normal bronchial epithelial cells	+(152.56)	+(33.52)

[a]Mean fluorescence intensity (MFI) was examined with the help of flow cytometric analysis after cell staining by using anti-Ep-CAM or anti-HLA-A24 monoclonal antibodies and FITC-labelled anti-mouse IgG $F(ab')_2$ fragments.

Table 2

These data demonstrated that Ep_{173}-specific CTLs kill tumour cells expressing both HLA-A24 and Ep-CAM.

We further examined whether C27 might recognize naturally processed peptides presented on the surfaces of tumour cells in the context of HLA-A24. Cytotoxicity of C27 against PC9 cells ($HLA\text{-}A24^+$ $Ep\text{-}CAM^+$ lung cancer cell line) was blocked by monoclonal antibodies specific to HLA-A24 or pan-class-I molecules, but not an anti-HLA-A2 monoclonal antibody (Fig. 4B), confirming the HLA-A24 restriction. Cold target inhibition assays demonstrated that C27-mediated cytotoxicity against PC9 cells was specifically inhibited in the presence of T2-A24 cells pre-pulsed with the cognate but not an irrelevant peptide (Fig. 4C), indicating that C27 recognized peptides that were naturally processed and presented.

Because Ep-CAM is expressed in some sites of normal epithelial cells, there has been concern about potential autoimmune reactions after *in vivo* activation of T cells specific to the molecule. We, therefore, tested an HLA-A*2402-positive, normal human bronchial epithelial cell line, designated as NHBE, with clone C27. As demonstrated in Fig. 6(A), C27 exerted toxicity, especially at higher effector : target ratios (40 : 1 and 20 : 1), which was specifically blocked by T2-A24 cells pre-pulsed with the cognate peptide (Fig. 6B). At lower effector : target ratios (10 : 1 and 5 : 1), however, C27-mediated lysis of NHBE was apparently lower than that of cancer cell lines, such as PC9, LU99, LC99A and HSC-2 (Fig. 5).

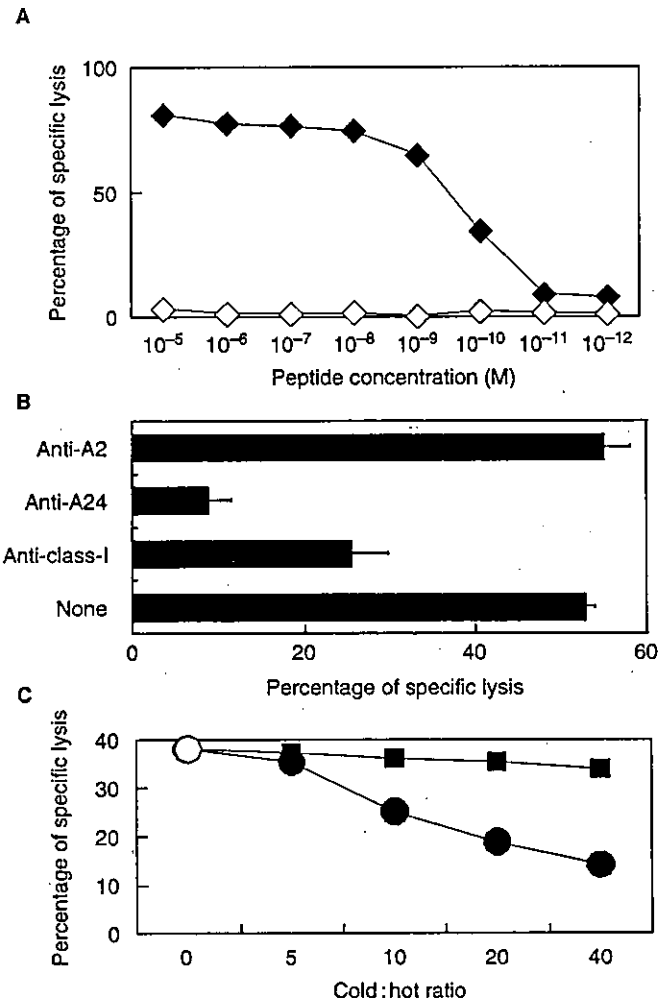

***Fig. 4.* Characterization of an Ep_{173}-specific cytotoxic T-lymphocyte (CTL) clone, C27.** An Ep_{173}-specific CTL clone, C27-mediated target cell (T2-A24) lysis in the presence of Ep_{173} (◆) and control peptide EBV-$LMP2_{419}$ (◇) has been shown (A). C27-mediated target cell (PC9) lysis was blocked with monoclonal antibodies specific to HLA-A24 and pan-class-I molecules (B). C27-mediated target cell (PC9) lysis was blocked with T2-A24 cells that had been loaded with Ep_{173} (●) but not with a control peptide EBV-$LMP2_{419}$ (■) (C). The cytotoxic assays were performed at effector to ^{51}Cr-labelled target ratios of 1, 10 and 5, in experiments shown in A, B and C, respectively. HLA, human leucocyte antigen.

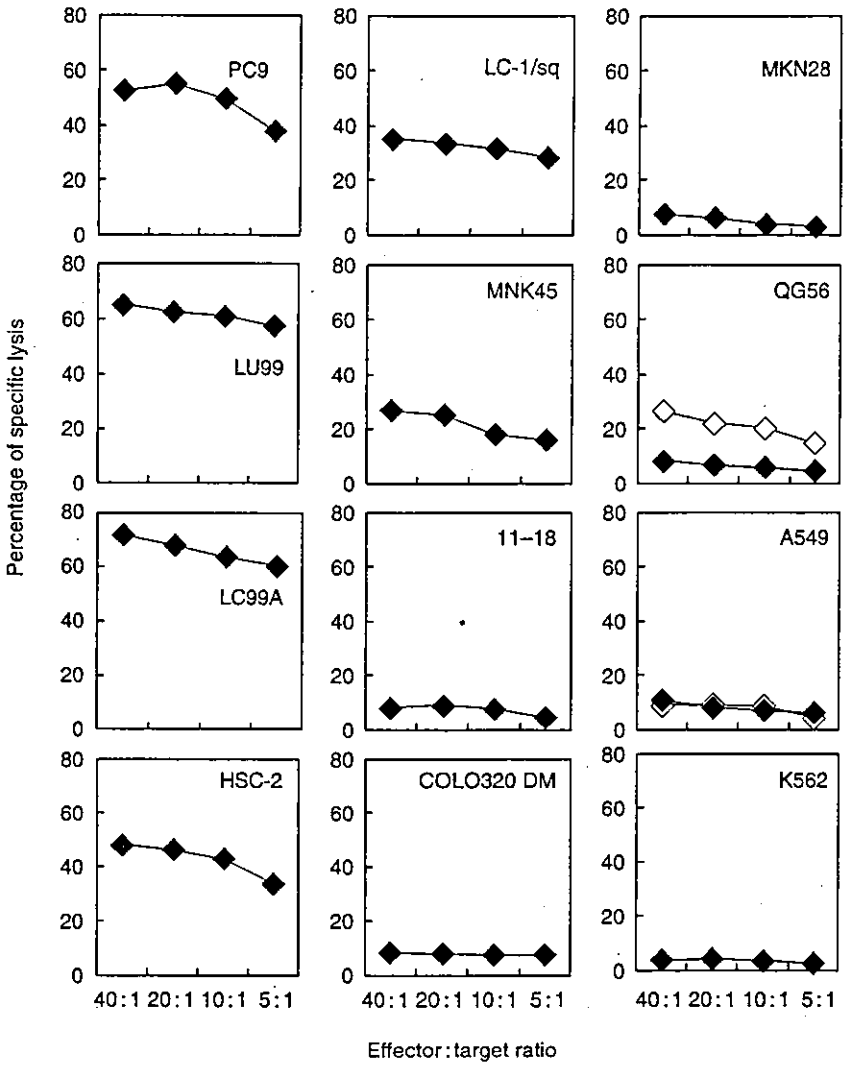

Fig. 5. **Cytotoxicity of the clone C27 against cancer cell lines.** An Ep_{173}-specific cytotoxic T-lymphocyte (CTL) clone, C27-mediated lysis of various cancer cell lines. Cell lines in the left and the centre columns express HLA-A24 on their surface, whereas those in the right do not. All the target cell lines except for 11–18, COLO320DM and A549 express the Ep-CAM. HLA-A*2402 transfectants of QG56 and A549 cells were also used as target cells (◇). K562 was included in order to assess natural killer-like cytotoxicity. HLA, human leucocyte antigen.

Discussion

According to recent findings, overexpression of Ep-CAM, a transmembrane protein mediating Ca^{2+}-independent cell–cell adhesion, is correlated with tumour grade (14, 34, 35), providing a useful marker for diagnosis of micrometastases (36) and a predictor of survival (14). The promoter region that regulates the Ep-CAM transcription has been cloned and it has shown to be negatively regulated by TNF-α (37).

Ep-CAM has become one of the major targets for immunotherapy with monoclonal antibody, because it is expressed in the vast majority of epithelial cell-derived cancers. The administration of Ep-CAM-specific murine monoclonal antibody (17-1A) into Duke's C colorectal cancer patients after surgery, for example, led to the prevention of distant metastasis and to prolonged survival after 7 years of follow-up evaluation (19). Recently, bispecific antibodies against Ep-CAM and CD3 (38, 39) or B7 (40) have been engineered in order to improve cytotoxicity with synergistic effects of antibodies and T lymphocytes. Considering the expression pattern, immunotherapy by using CTL directed against Ep-CAM appears attractive. Indeed, Ep-CAM peptides that elicited cellular immune responses restricted by HLA-A*0201 have recently been identified (4, 20). Of note, a natural T-cell response against Ep-CAM has been observed in colorectal cancer patients (41, 42). The available evidence, thus, suggests the possibility of therapeutic approaches using Ep-CAM as a target of CTLs.

We, in this study, explored immunogenic peptides derived from Ep-CAM that can elicit cellular immune responses against Ep-CAM$^+$ tumour cells in the context of HLA-A24, which is positive in nearly 20% of persons of European descent and more than 60% of Japanese. For that purpose, seven candidate peptides were first predicted from the Ep-CAM protein with a bioinformatic approach. Secondly, MHC

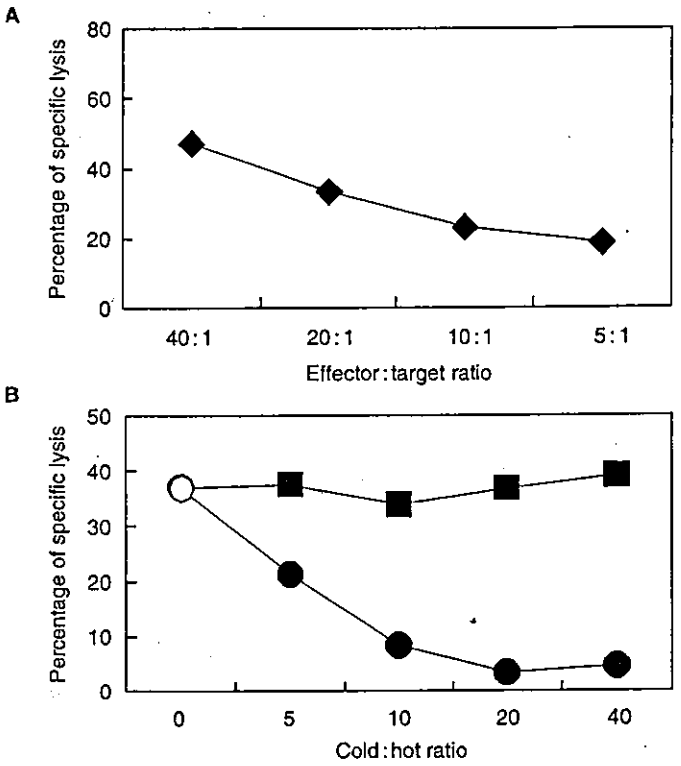

Fig. 6. **Cytotoxicity of the clone C27 against normal bronchial epithelial cells.** C27-mediated lysis of normal bronchial epithelial cells (NHBE) expressing HLA-A24 and Ep-CAM (A) and C27-mediated NHBE cell lysis were blocked with T2-A24 cells pulsed with Ep_{173} (●) but not with a control peptide EBV-$LMP2_{419}$ (■) (B). The effector to hot target ratio was 20. HLA, human leucocyte antigen.

stabilization assays were used in order to determine the binding affinity of the peptides with HLA-A*2402 molecules, revealing one peptide, Ep_{173} (RYQLDPKFI), to have the highest affinity and Ep_{304} (EMGEMHREL) the lowest. Thirdly, we successfully established Ep_{173}-specific polyclonal CTL lines from four of five healthy donors. A CTL clone, C27, demonstrated fine specificity for Ep_{173}, which was naturally presented on the surfaces of tumour cells, because C27-mediated PC9 cell lysis was blocked by both anti-HLA-A24 monoclonal antibody and Ep_{173}-pulsed cold target cells. An Ep_{250}-specific polyclonal CTL line was generated from donor 3, raising the possibility that Ep_{250} is another CTL epitope presented by HLA-A*2402. We, so far, could not establish CTL clones specific to Ep_{250}, which are required for further evaluation of the peptide.

It is of note that there is no correlation between the level of expression of Ep-CAM and the degree of CTL-mediated lysis (Figs. 3 and 5; Table 2). The level of HLA expression does not seem to explain the issue. We speculate that the Ep-CAM may be more resistant to proteasome degradation in cells with high Ep-CAM expression and low or moderate sensitivity to CTL-mediated lysis (LC-1/sq, MKN45 and QG56). On the contrary, there may be a higher turnover rate of the Ep-CAM in cells with low expression and high sensitivity to CTL-mediated lysis (LU99, LC99A and HSC-2).

Although Ep-CAM is a candidate target for both humoral and cellular immunotherapy, a persistent difficulty arises because of its presentation on normal epithelial tissues. Indeed, the CTL clone, C27, lysed HLA-A24-positive normal bronchial epithelial cells in an epitope-specific fashion, especially at high effector : target ratios (Fig. 6). However, at lower effector : target ratios, such as 10:1 and 5:1, C27-mediated lysis of NHBE was apparently lower than that of C27-sensitive cancer cell lines (Fig. 5). In addition, C27-mediated lysis of NHBE was more efficiently inhibited by T2-A24 cells pulsed with the cognate peptide (Fig. 6B) than that of the PC9 lung cancer cell line (Fig. 4C), implicating a lower density of HLA-A*2402/Ep_{173} complexes on the surface of NHBE cells.

It has been reported that an HLA-A*0201-restricted Ep-CAM-specific CTL line could not lyse normal bronchial epithelial cells under conditions, whereby epithelial tumour cells were efficiently killed (20). So far, immunization of colorectal carcinoma patients with a recombinant canarypox virus expressing Ep-CAM has been well tolerated and has been shown to induce anti-Ep-CAM CTL responses without causing autoimmune reactions (43). Interestingly, a monoclonal antibody specific to Ep-CAM did not localize to Ep-CAM-positive normal tissues of human Ep-CAM transgenic mice (6), indicating limited *in vivo* accessibility, which might explain any absence of autoimmunity. Another example of vaccination of tissue-specific self-antigen is provided by means of immunotherapy targeting CEA (carcino embryonic antigen). Greiner et al. (44) reported that vaccination of CEA-transgenic mice with a recombinant canarypox virus expressing CEA can generate substantial anti-tumour immunity with little or no autoimmunity. They proposed possible explanations including (i) differential susceptibility of tumour and normal tissues to the immune effector arms and (ii) blockage of the autoreactive T-cell activity by tolerizing antigen-presenting cells or the presence of regulatory T cells in order to terminate the response in the normal epithelia. Further studies are required in order to evaluate potential autoimmune reactions with Ep_{173} immunization. Littermates of human Ep-CAM transgenic mice (6) bred with HLA-A*2402 transgenic mice would provide a suitable model to study this issue.

In conclusion, we present, in this study, a novel HLA-A*2402-restricted epitope, Ep_{173} (RYQLDPKFI), which has the ability to induce $CD8^+$ T cells with high-affinity antigen receptors directed to HLA-A*2402/Ep_{173} complexes. All the data suggest that the epitope-specific CTL responses may play some roles in both anti-cancer and autoimmune reactions. The peptide should prove useful to study anti-Ep-CAM CTL responses among populations possessing HLA-A*2402.

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INTERFERON-γ DIFFERENTIALLY REGULATES SUSCEPTIBILITY OF LUNG CANCER CELLS TO TELOMERASE-SPECIFIC CYTOTOXIC T LYMPHOCYTES

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There is accumulating evidence that peptides derived from the catalytic subunit of human telomerase reverse transcriptase (hTERT) are specifically recognized by $CD8^+$ cytotoxic T lymphocytes. We investigated the cytotoxicity of a human leukocyte antigen (HLA)-A*2402-restricted hTERT-derived peptide 461–469 ($hTERT_{461}$)-specific $CD8^+$ T-cell clone, designated as K3-1, established from a healthy donor by repetitive peptide stimulation. This clone exhibited cytotoxicity against 4 out of 6 HLA-A24-positive lung cancer cell lines with positive telomerase activity but not 4 HLA-A24-negative examples. When the target cells were pretreated with 100 U/ml of interferon (IFN)-γ for 48 hr, the susceptibility to K3-1 increased with PC9 cells but unexpectedly decreased with LU99 cells. However, in both cell lines, the expression of molecules associated with epitope presentation such as HLA-A24, transporters associated with antigen processing, low molecular weight polypeptide 7 and proteasome activator 28 was similarly increased after IFN-γ treatment. Results of CTL assays using acid-extracted peptides indicated that the epitope increased on PC9 cells but not on LU99 cells after IFN-γ treatment. Semi-quantitative reverse transcriptase polymerase chain reaction disclosed that the expression of hTERT was attenuated in LU99 but not in PC9 cells, accounting for the decreased cytotoxicity mediated by K3-1. The attenuation of the hTERT expression and K3-1-mediated cell lysis after IFN-γ treatment was also observed in primary adenocarcinoma cells obtained from pulmonary fluid of a lung cancer patient. Our data underline the utility of peptide $hTERT_{461}$ in immunotherapy for lung cancer, as with other malignancies reported earlier, and suggest that modulation of hTERT expression by IFN-γ needs to be taken into account in therapeutic approach.

Key words: *telomerase; hTERT; immunotherapy; lung cancer; Interferon-γ*

Human chromosomes terminate with 5–15 kilobases of repetitive telomeric DNA $(TTAGGG)_n$,[1] which protect against DNA degradation, end-to-end fusion, rearrangements and chromosome loss.[2] In normal cells, such as cultured skin fibroblasts, telomeric DNA becomes shortened with every round of replication,[3] ultimately leading to replicative senescence. In contrast, with permanently established cell lines from malignant tumors, telomeres are believed to be elongated by a unique ribonucleoprotein enzyme, called telomerase, which adds telomeric sequences *de novo*.[1] Indeed, there is clear evidence that telomerase activity is involved in tumorigenesis.[4,5] Normal tissues display little or no telomerase activity, and activation of the enzyme may therefore play a critical role in cell immortalization.

Human telomerase complexes are composed of telomerase RNA component,[6] telomerase protein 1[7,8] and hTERT.[9] Messenger RNA expression of hTERT is essential for telomerase activation during cellular immortalization and tumor progression,[9] and ectopic expression of the hTERT gene in telomerase-negative cells can induce telomerase activity to levels comparable to those in immortal telomerase-positive cells.[10] The expression of hTERT has been frequently demonstrated in telomerase-positive primary tumors and cancer cell lines but found to be low or undetectable in normal tissues.[9–13] Thus, hTERT could be a candidate universal tumor antigen for immunotherapy and vaccine approaches.

Several studies have been conducted to test the possibility that hTERT could serve as a tumor antigen recognized by specific CTL. Indeed, hTERT peptide-specific CTL have proved cytotoxic to cell lines derived from various malignancies including leukemias,[14,15] osteosarcoma, ovarian carcinoma, non-Hodgkin's lymphoma,[15] multiple myeloma,[15,16] melanoma[15,17] and cancers of breast, colon, lung,[17] prostate[17,18] or kidney.[18] Recent studies revealed that hTERT is expressed in 89%[13] to 93.9%[19] of primary lung cancers.

In our study, we first asked the question whether hTERT-specific CTL recognize and kill lung cancer cells applying an HLA-A*2402-restricted hTERT-derived peptide ($hTERT_{461}$)-specific $CD8^+$ T-cell clone, generated from a healthy donor, and a panel of lung cancer cell lines with positive telomerase activity as targets. The findings confirm and extend previous results, supporting the feasibility of developing CTL-based immunotherapy targeting hTERT in some, if not all, lung cancer patients. In addition, interesting evidence was obtained to demonstrate that IFN-γ treatment of the target cells did not always enhance CTL recognition.

Abbreviations: CD40-B, CD40-activated B; CTL, cytotoxic T lymphocyte; FITC, fluorescein isothiocyanate; hTERT, human telomerase reverse transcriptase; IRF, interferon regulatory factor; HLA, human leukocyte antigen; IL, interleukin; LCL, lymphoblastoid B-cell line; LMP, low molecular weight polypeptide; MAb, monoclonal antibody; MHC, major histocompatibility complex; PBMC, peripheral blood mononuclear cell; PE, phycoerythrin; RT-PCR, reverse transcription polymerase chain reaction; TAP, transporters associated with antigen processing.

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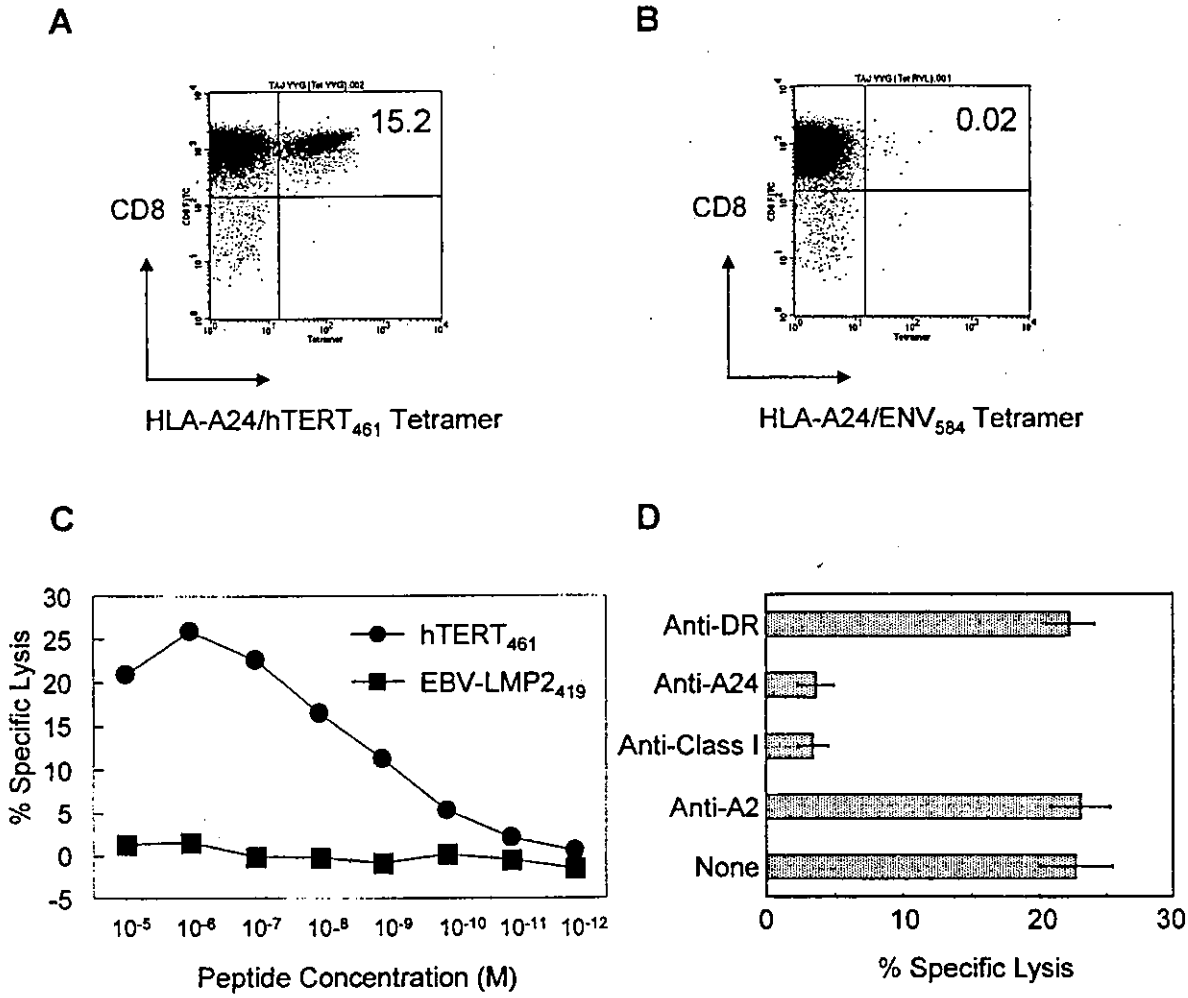

FIGURE 1 – Characterization of polyclonal CTL specific to $hTERT_{461}$. Polyclonal $CD8^+$ T cells after stimulation 4 times were stained with FITC-labeled anti-CD8 antibodies and PE-labeled HLA-A24-tetramers incorporating $hTERT_{461}$ (*a*) or a control peptide, ENV_{584} (*b*), and analyzed by flow cytometry. The percentages of tetramer-positive cells in total $CD8^+$ T cells are shown. (*c*) Results of CTL assays using serial dilutions of $hTERT_{461}$ (closed circle) and an EBV-derived control peptide, $EBV\text{-}LMP2_{419}$ (closed square). Cytotoxicity of polyclonal CTL to T2-A24 cells in the presence of indicated concentrations of each peptide was determined by ^{51}Cr release assays at an effector-target ratio of 1. (*d*) Inhibitory effect of an anti-HLA class I monoclonal antibody or an anti-HLA-A24 monoclonal antibody on cytotoxicity of a CTL clone K3-1 against a HLA-A24-positive cell line, PC9. Chromium-labeled target cells were incubated with either monoclonal antibodies specific to HLA class I, HLA-A24, HLA-A2 or HLA-DR molecules, before addition of K3-1 cells. The cytotoxic assays were done at an effector-target ratio of 10.

MATERIAL AND METHODS

Donors and cell lines

Peripheral blood mononuclear cells (PBMC) were isolated from 4 HLA-A24-positive healthy donors by centrifugation on a Ficoll density gradient. Epstein-Barr virus (EBV)-transformed LCL were established as previously described[20] and cultured in RPMI 1640 medium (Sigma Chemical Co., St. Louis, MO) supplemented with 10% fetal calf serum (FCS) (Life Technologies Limited, Auckland, NZ), 2×10^{-3} M L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 100 μg/ml kanamycin and 5×10^{-5} M β-mercaptoethanol (referred to as complete medium). CD40-activated B (CD40-B) cells were generated using NIH-3T3-hCD40 ligand cells (kindly provided by Dr. G. Freeman, Dana-Farber Cancer Institute, Boston, MA) as previously described.[21,22] Pulmonary fluid was obtained from an HLA-A24-positive patient with lung adenocarcinoma for primary culture of the cancer cells. The study design and purpose, which had been approved by the institutional review board of Aichi Cancer Center, were explained fully to all donors. Samples were obtained after informed consent was confirmed.

Human lung cancer cell lines, LC-1/sq and LU99 cells, were purchased from the Japanese Collection of Research Bioresources (Tokyo, Japan) and RIKEN Cell Bank (Tsukuba, Japan), respectively. LC-1/sq cells were maintained in 45% RPMI1640 medium and 45% Ham's F12 (Sigma Chemical Co.) supplemented with 10% FCS, L-glutamine, penicillin, streptomycin and kanamycin. All other lung cancer cell lines (LU99, PC9, 11-18, LC99A, LC65A, LK79, A549, QG56 and RERF-LC-MT) and a chronic megakaryoblastic leukemia cell line, MEG-01, were maintained in the complete medium. K562 cells were maintained in IMDM (Sigma Chemical Co.) supplemented with 10% FCS, L-glutamine, penicillin, streptomycin and kanamycin. HLA-A*2402-trans-

TABLE 1—TELOMERASE ACTIVITY AND HLA-A24 EXPRESSION OF TARGET CELL LINES USED IN THIS STUDY

Cells	Tumor origin	Telomerase activity[1]	Surface HLA-A24 expression[2]
Lung cancer cell lines			
PC9	Adenocarcinoma	+	+
11–18	Adenocarcinoma	+	+
LC-1/sq	Squamous cell carcinoma	+	+
LU99	Giant cell carcinoma	+	+
LK79	Small cell carcinoma	+	+
LC99A	Large cell carcinoma	+	+
LC65A	Small cell carcinoma	+	−
RERF-LC-MT	Adenocarcinoma	+	−
A549	Adenocarcinoma	+	−
QG56	Squamous cell carcinoma	+	−
Hematopoietic cell lines[3]			
MEG-01	Leukemia	+	+
K562	Leukemia	+	−
T2-A24	—	Not done	+

[1]Telomerase activity was detected as described in the Material and Methods.–[2] To detect surface expression of HLA-A24 molecules, cells were stained with an anti-HLA-A24 antibody and subsequently with FITC-labeled anti-mouse IgG $F(ab')_2$ fragments and analyzed by flow cytometry.–[3]MEG-01, a control cell line expressing telomerase and HLA-A24 molecules; K562, a representative cell line susceptible to natural killer-like cytotoxicity; T2-A24, a TAP-deficient cell line expressing HLA-A24 molecules.

fected, TAP-negative T2-A24 cells[23] were cultured in complete medium containing 0.8 mg/ml of G418 (Gibco, Grand Island, NY). Pulmonary fluid containing adenocarcinoma cells was diluted with the complete medium and cultured in the presence or absence of IFN-γ for 48 hr. After the incubation, adherent cells were used for RT-PCR analysis and as target cells for hTERT-specific CTL.

A retrovirus encoding HLA-A*2402 was infected into the HLA-A24-negative cell lines, QG56 and A549, as previously described.[24] The infected cells were maintained in complete medium with puromycin at the final concentration of 0.6 (for QG56) or 0.9 (for A549) μg/ml for selection and designated as QG56–A24 and A549–A24, respectively.

Peptides

Two HLA-A24-restricted CTL epitope peptides derived from hTERT,[14] VYAETKHFL (residues 324–332, designated as $hTERT_{324}$) and VYGFVRACL (residues 461–469, designated as $hTERT_{461}$), a human immunodeficiency virus-1 (HIV-1) envelop peptide RYLRDQQLL[25] (residues 584–592, designated as ENV_{584}) and an EBV latent membrane protein 2 peptide TYGPVFMCL[20] (residues 419–427, designated as $EBV\text{-}LMP2_{419}$) were synthesized by Toray Research Center (Kamakura, Japan).

Cell staining and flow cytometric analysis

Surface expression of HLA-A24 molecules was examined by indirect immunofluorescence using an HLA-A24 MAb (One Lambda, Inc. Canoga Park, CA) and FITC-labeled anti-mouse IgG $F(ab')_2$ fragments (IMMUNOTECH, Marseilles, France). MHC-tetramers were produced as previously described.[23,26] $CD8^+$ T cell lines were stained with PE-labeled HLA-A*2402-tetramers incorporating $hTERT_{324}$, $hTERT_{461}$ or ENV_{584}. Flow cytometric analysis of the stained cells was performed using a FACSCalibur (Becton Dickinson, San Jose, CA) and the data were analyzed using CellQuest software (Becton Dickinson).

Reverse transcription polymerase chain reaction (RT-PCR)

Total RNA was extracted from cultured cell lines. Gene-specific oligonucleotide primers were synthesized at Proligo (Kyoto, Japan) and used to evaluate the mRNA expression pattern of hTERT,[13] TAP-1, TAP-2[27] and IRF-1.[28] RT-PCR was performed using a thermal cycler (Perkin-Elmer, Wellesley, MA) and the products were analyzed by 1.5% gel electrophoresis and ethidium bromide visualization.

Western blot analysis

Western blot analysis was performed as described previously[29] with slight modifications. Briefly, cells were lysed in lysis buffer (50 mM Tris/HCl, pH 7.5, 5 mM $MgCl_2$, 1 mM EDTA, 0.5% Triton X-100, 10 μM leupeptin, 2.8 μM pepstatin and 0.85 mM phenylmethanesulfonyl fluoride) for 30 min at 4°C. The post-nuclear supernatant was quantified by absorbance at 280/260 nm for protein concentrations, and aliquots of 130 μg protein were applied to 12% SDS-PAGE. The proteins were blotted onto Immobilon-P membranes (Millipore Corporation, Bedford, MA), blocked with PBS containing 10% low fat dry milk and 0.1% Tween-20 overnight at 4°C and probed with rabbit polyclonal Abs specific to low molecular weight polypeptide 7 (LMP7) and proteasome activator 28 (PA28) α subunits (Affinity, Mamhead, U.K.) followed by peroxidase-conjugated goat anti-rabbit IgG (Zymed, San Francisco, CA). Proteins were visualized using an ECL Western blot detection system (Amersham Biosciences, Buckinghamshire, UK).

Generation of hTERT-specific polyclonal and clonal CTL using peptide-pulsed CD40-B cells as antigen presenting cells

CD40-B cells (2.5×10^5) were pulsed with $hTERT_{324}$ or $hTERT_{461}$ at 1×10^{-5} M for 1 hr and irradiated at 33 Gy. $CD8^+$ T cells (1×10^6) were isolated from donated PBMC with the aid of CD8 MicroBeads (Miltenyi Biotec, Bergisch Gladbach, Germany) and cocultured with autologous peptide-pulsed CD40-B cells in 2 ml culture medium in the presence of 25 ng/ml IL-7 (Genzyme, Cambridge, MA) and 5 ng/ml IL-12 (R&D Systems, Minneapolis, MN) at 37°C in a 5% CO_2 incubator. On days 7, 14, 21 and 28, $CD8^+$ T cells were restimulated with peptide-pulsed and γ-irradiated CD40-B cells. One day after each restimulation, human recombinant IL-2 (Takeda Chemical Industries, Osaka, Japan) was added to a final concentration of 20 U/ml. If necessary, rapidly growing cells were split into 2 to 3 wells and fed with fresh culture medium containing 20 U/ml of IL-2. Specificity of the T cells was examined with tetramer staining and cytotoxic assays. To establish T-cell clones, limiting dilution of the polyclonal CTL was performed.[23] Briefly, polyclonal $CD8^+$ T cells were seeded at 1 or 3 cells/well in round-bottomed 96-well plates containing the culture medium (0.2 ml) with anti-CD3 MAb (30 ng/ml, Ortho Diagnostics, Raritan, NZ), IL-2 (30 U/ml), γ-irradiated (33 Gy) 1×10^5 PBMC and γ-irradiated (55 Gy) 2×10^4 LCL. After 2 weeks of culture, growing cells positively stained for the HLA-A*2402/$hTERT_{461}$-tetramer were transferred into flasks and expanded as above.

CTL assay

Target cells were labeled with chromium (^{51}Cr) in 100 μl culture medium for 1 h at 37°C. In some experiments, predetermined amounts of blocking antibodies, W6/32 (anti-HLA class I), MA2.1 (anti-HLA-A2), A11.1 (anti-HLA-A24) and HDR-1 (anti-HLA class II) were added to the wells 30 min before adding

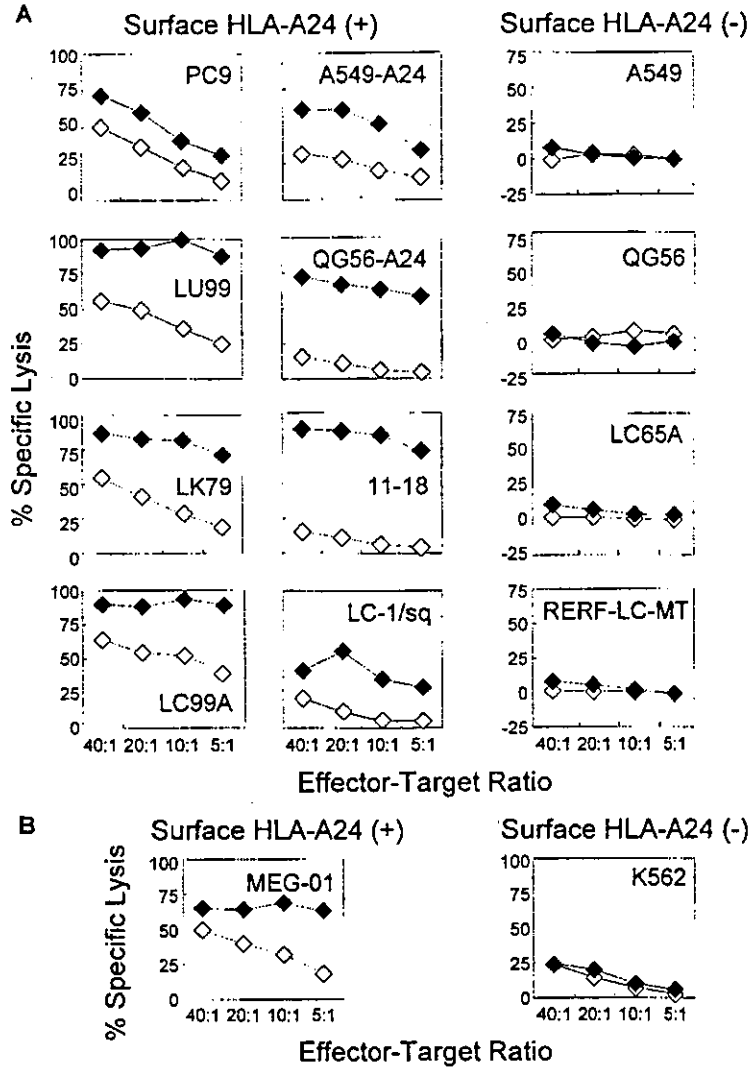

FIGURE 2 – Cytotoxicity of $hTERT_{461}$-specific CTL clone, K3-1 against cancer cell lines. (a) Cytotoxicity of $hTERT_{461}$-specific CTL clone K3-1 for 8 lung cancer cell lines with positive surface HLA-A24 expression and 4 with negative surface HLA-A24 expression as target cells (see Table I). The HLA-A*2402 gene was retrovirally transfected into A549 and QG56 cells, and the resultant transfectants designated as A549-A24 and QG56-A24, respectively. Assays were performed in the presence (closed diamond) or absence (open diamond) of 1×10^{-7} M $hTERT_{461}$ at the indicated effector-target ratios. (*b*) Cytotoxicity of $hTERT_{461}$-specific CTL clone K3-1 against MEG-01, a control hematopoietic cell line expressing telomerase and HLA-A24 molecules, and K562, a representative cell line susceptible to natural killer cytotoxicity, as target cells. Assays were performed in the presence (closed diamond) or absence (open diamond) of 1×10^{-7} M $hTERT_{461}$ at the indicated effector-target ratios.

effector cells to determine the HLA restriction. In others, target cells were treated with 100 U/ml of IFN-γ for 48 hr before chromium labeling. The plates were incubated for 5 hr at 37°C, and the supernatants were counted in a gamma counter. The percentage specific ^{51}Cr release was calculated as follows: 100 × (experimental release − spontaneous release)/(maximum release − spontaneous release).

Extraction of naturally processed peptides from cell lines

Isolation of peptides from cell cultures was performed as previously described[30] with slight modifications. Briefly, confluent PC9 or LU99 cells (either treated or untreated with IFN-γ for 48 hr) in T225 flasks (Costar, Cambridge, MA) were washed 3 times with PBS and incubated with 5 ml of citrate-phosphate buffer (pH 3.3) for 1 min. The buffer containing eluted peptides was harvested and stored at −80°C until use. Peptides were repetitively stripped for 4 consecutive days.

The acid-extracted peptides were filtered and concentrated on SepPak Light C18 Cartridges (Waters Corporation, Milford, MA) according to the manufacturer's instructions. Bound peptides were eluted with 80% acetonitrile and 0.1 % trifluoroacetic acid, concentrated in a Speed-Vac (Savant Instruments, Inc., Hicksville, NY) and pulsed on ^{51}Cr-labeled T2-A24 cells. K3-1-mediated target cell lysis was assessed as described above.

Measurement of telomerase activity

Telomerase activity was measured by the telomeric repeat amplification protocol using Telo TAGGG Telomerase PCR ELISA^{PLUS} (Roche Diagnostics Corporation, Indianapolis, IN) according to the manufacturer's instruction. Samples were considered as telomerase-positive if the difference in absorbance (absorbance of sample − absorbance of heat-treated sample) was more than 2-fold background activity, according to the protocol supplied with the reagents.

RESULTS

Generation of hTERT peptide-specific $CD8^+$ CTLs

To generate hTERT-specific $CD8^+$ T cell lines, $CD8^+$ T cells of 4 HLA-A24-positive healthy donors were stimulated weekly with autologous CD40-B cells pulsed with either of the HLA-A*2402-restricted hTERT-derived peptides, $hTERT_{324}$ or $hTERT_{461}$. After