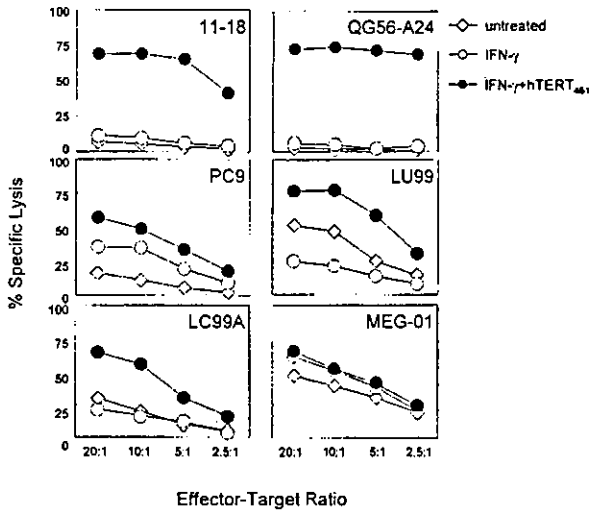


FIGURE 3 – K3-1-mediated lung cancer cell lysis induced by IFN-γ treatment. Cytotoxicity of the $hTERT_{461}$-specific CTL clone K3-1 against HLA-A24-positive lung cancer cells determined with (open circle) or without (open diamond) IFN-γ pre-treatment (100 U/ml for 48 hr). Cytolysis of IFN-γ treated cells was also tested in the presence of 1×10^{-7} M $hTERT_{461}$ (closed circle). Assays were performed at the indicated effector-target ratios.

the fourth stimulation, the T-cell lines were stained with HLA-A24-tetramers incorporating $hTERT_{324}$, $hTERT_{461}$ or ENV_{584}. A T cell line from a donor stimulated with $hTERT_{461}$ was specifically stained with HLA-A24-tetramers incorporating $hTERT_{461}$ but not ENV_{584} (15.2% *vs.* 0.02% in total CD8+ cells, Fig. 1*a,b*). This line showed cytotoxicity to T2-A24 cells pulsed with $hTERT_{461}$ dose-dependently but not with control peptide $EBV\text{-}LMP2_{419}$ (Fig. 1*c*). None of the other polyclonal T-cell lines were stained with HLA-A24-tetramers incorporating peptides used for individual stimulation, even after a fifth stimulation (data not shown).

A $CD8^+$ CTL clone was established by limiting dilution of the polyclonal T-cell line and designated as K3-1. The integrity of K3-1 was assessed with the HLA-A24/$hTERT_{461}$-tetramer (data not shown).

Lysis of lung cancer cell lines by the $hTERT_{461}$ peptide-specific CTL clone, K3-1

We next examined K3-1-mediated cytotoxicity against a panel of lung cancer cell lines (Table I). Among the 10 lung cancer cell lines examined, 6 were positive for HLA-A24 expression, and all cell lines featured telomerase activity (Table I). Results for cytotoxicity are summarized in Figure 2*a*, only HLA-A24-positive lung cancer cell lines (PC9, LU99, LK79 and LC99A) being affected. The degree of cell lysis was comparable to that observed for a leukemia cell line, MEG-01 cells (Fig. 2*b*), previously reported to be well recognized by HLA-A24-restricted hTERT-specific CTL.[14] The cytotoxicity of K3-1 against PC9 cells was blocked by an anti-HLA-A24 MAb, but not anti-HLA-A2 or HLA-DR MAbs showing HLA-A24 restriction (Fig. 1*d*). The K3-1-mediated cytotoxicity against PC9 cells was specifically inhibited by the presence of T2-A24 cells pre-pulsed with the cognate but not an irrelevant peptide (data not shown), indicating K3-1 could recognize $hTERT_{461}$ naturally processed and presented on the surfaces of the target cells. As shown in Figure 2*a* (center column), some HLA-A24-positive and HLA-A24-transfected lung cancer cell lines were not effectively lysed by K3-1 despite confirmation of surface expression of HLA-A24 by indirect immunofluorescence and flow cytometry. However, the cytotoxicity against these cell lines was enhanced in the presence of $hTERT_{461}$ in the medium, suggesting insufficient epitope density on these cells.

HLA-A24-negative cell lines, A549, QG56, LC65A and RERF-LC-MT cells were not lysed at all by K3-1 either in the presence or absence of the cognate peptide (Fig. 2*a*, right column). K562 cells were included to assess the degree of NK-like cytotoxicity of K3-1 cells, which turned out to be negligible (Fig. 2*b*).

K3-1-mediated lung cancer cell lysis after IFN-γ treatment

Pretreatment of target cells with IFN-γ is well known to augment epitope processing and presentation.[30] Thus, we asked the question whether IFN-γ treatment augments CTL-mediated cell lysis of the cytolysis-negative cell lines (Fig. 2*a*, center column) by improved epitope processing and presentation.[30] The cytolysis-positive cell lines were also tested. As demonstrated in Figure 3, there was no augmentation of K3-1-mediated lysis in the 11–18 and QG56-A24 cases. Lysis of LC-1/sq and A549-A24 cells was also not augmented by IFN-γ treatment (data not shown). Of note, K3-1-mediated lysis of PC9, LU99, LC99A and MEG-01 cells was differentially affected by IFN-γ pretreatment (Fig. 3). Thus the lysis of PC9 and MEG-01 cells was increased by the treatment, but with LC99A cells, it was unchanged or slightly decreased. Most interestingly, the lysis of LU99 cells was clearly reduced by the IFN-γ treatment.

IFN-γ induces gene expression of components involved in antigen processing and presentation in the lung cancer cells

Unexpectedly, IFN-γ affected K3-1-mediated lysis differently on PC9, LU99, LC99A and MEG-01 cells (Fig. 3). Therefore, we examined whether there is any difference of expression pattern of molecules important for class I antigen presentation. First, HLA-A24 expression was studied and found to be increased after IFN-γ treatment in all the cell lines (Fig. 4*a*). Second, expression of TAP-1 and TAP-2 was studied using semi-quantitative RT-PCR, mRNAs of both being also consistently increased after the treatment (Fig. 4*b*). Third, the expression of the LMP7, 1 of the 3 catalytic subunits of immunoproteasomes, and PA28, a regulator of the immunoproteasome, was studied using Western blotting with specific MAbs. In all the cell lines but QG56-A24, where the expression did not change, both proteins were increased after the treatment (Fig. 4*c*). In summary, we could not detect any difference in expression patterns of these molecules to account for the differential influence of IFN-γ.

Differential susceptibility of lung cancer cell lines to cytotoxicity of the CD8+ CTL clone, K3-1

To disclose the differential susceptibility to K3-1 in more detail, we compared cytotoxicity against 2 lung cancer cell lines, PC9 cells whose lysis was increased by IFN-γ and LU99 cells whose lysis was decreased, in the presence of a wide range of cognate peptide concentrations. After IFN-γ treatment, PC9 cells were efficiently lysed by K3-1 with any concentration of the peptide (Fig. 5*a*). In contrast, PC9 cells without IFN-γ treatment and LU99 cells, irrespective of IFN-γ treatment, demonstrated exogeneous peptide dose-dependent K3-1-mediated cell lysis, which was similar to the results using T2-A24 cells as target cells (compare Fig. 5*a* and Fig. 1*c*). These observations strongly suggest that the epitope density is saturated on the PC9 cells after IFN-γ treatment but not on the PC9 cells without the treatment and LU99 cells either with or without IFN-γ treatment.

In addition, to confirm that the epitope density was increased in PC9 but not LU99 cells after IFN-γ treatment, naturally processed peptides were acid-eluted from the cells, concentrated and tested by K3-1 after pulsing on T2-A24 cells applying ^{51}Cr-release assays. The results demonstrated in Figure 5*b* indicate elevation in the epitope peptides on the surfaces of PC9 but not LU99 cells after the IFN-γ treatment.

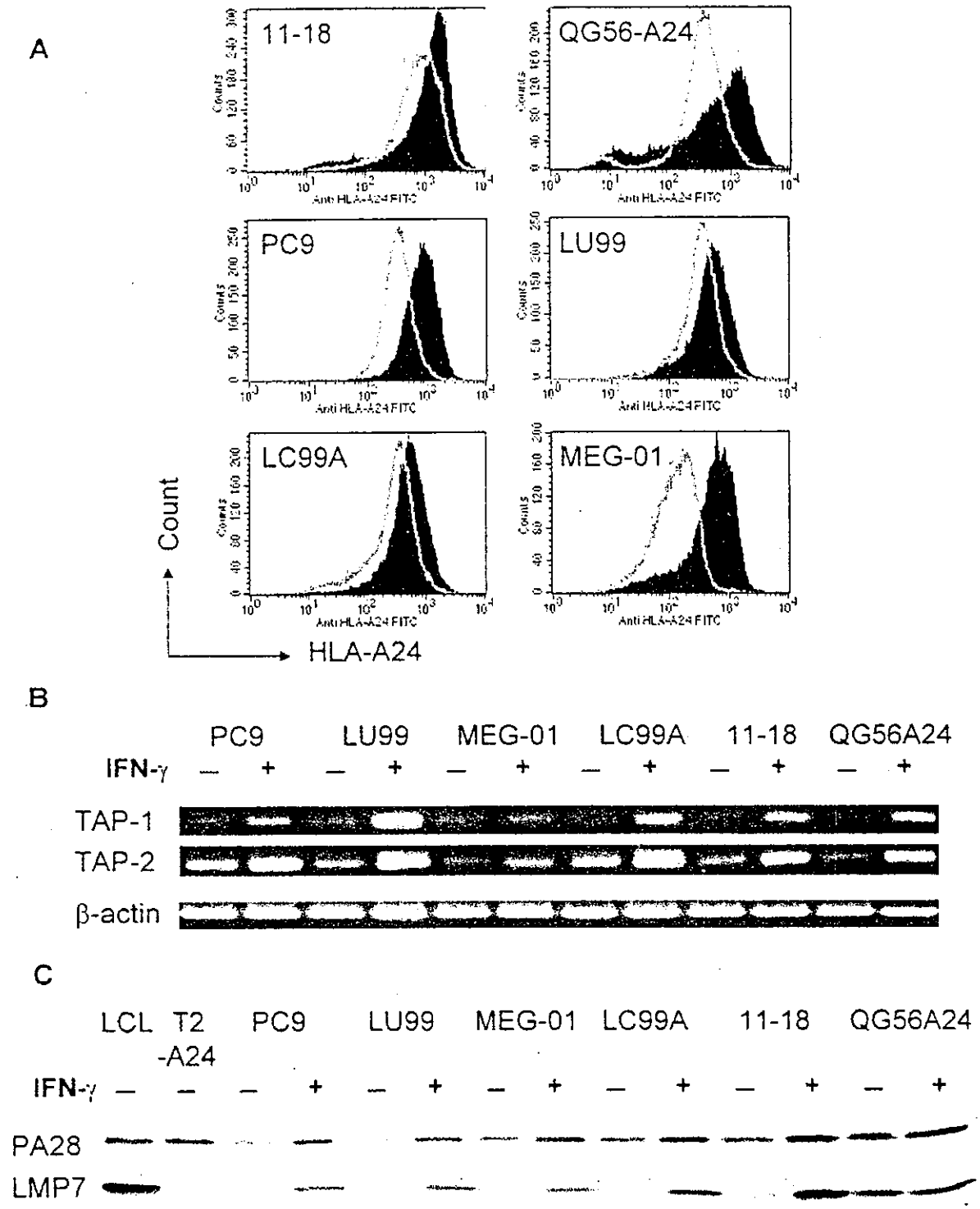


FIGURE 4 – Effects of IFN-γ on the regulation of molecules which play roles in antigen processing and presentation. (*a*) Results for surface HLA-A24 expression with (black shadowed) and without (lined) IFN-γ treatment analyzed by flow cytometry. Surface expression of HLA-A24 molecule was examined by indirect immunofluorescence using an HLA-A24 MAb and FITC-labeled anti-mouse IgG $F(ab')_2$ fragments. (*b*) Results of semi-quantitative RT-PCR analysis of TAP-1, and -2. Primers specific for TAP-1, and -2, as well as β-actin as a control were used for amplification of mRNA from cancer cell lines either treated or untreated with IFN-γ. (*c*) Results of Western blot analysis of PA28 and LMP7 molecules. Samples were obtained before and after treatment of cancer cells with IFN-γ.

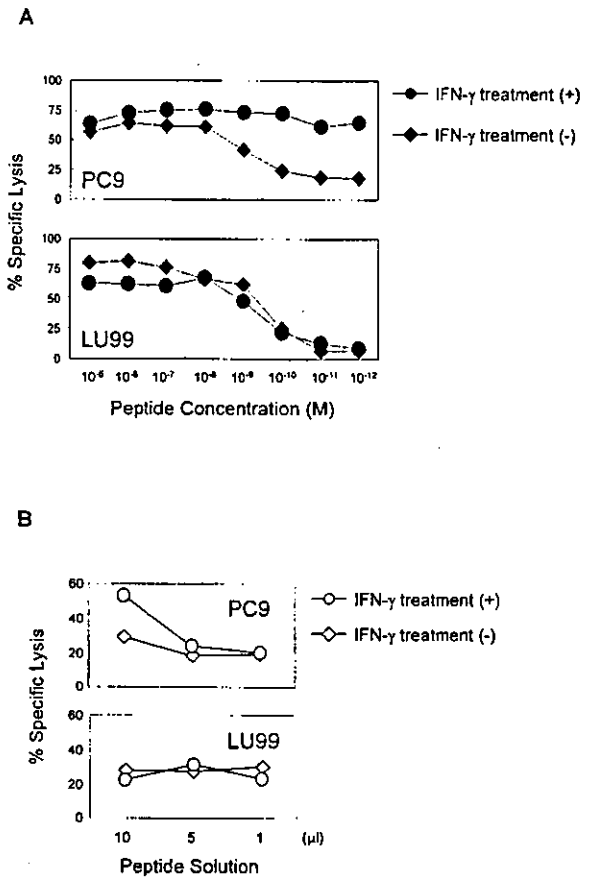


FIGURE 5 – The effects of IFN-γ on susceptibility of PC9 and LU99 cell lines to K3-1. (*a*) Cytotoxicity of the $hTERT_{461}$-specific CTL clone K3-1 against HLA-A24-positive lung cancer cells, PC9 or LU99, either treated (closed circle) or untreated (closed diamond) with 100 U/ml IFN-γ for 48 hr, as determined in ^{51}Cr release assay. The assay was performed in the presence of the indicated concentrations of the peptide $hTERT_{461}$ at an effector-target ratio of 5. (*b*) Naturally processed peptides were isolated from PC9 and LU99 cells, either treated (open circle) or untreated (open diamond) with IFN-γ, and concentrated. Indicated volumes of the peptide solution were pulsed on ^{51}Cr-labeled T2-A24 cells. K3-1-mediated target cell lysis was assessed at an effector-target ratio of 5.

Down-regulation of hTERT expression induced by IFN-γ

Very recently, Lee *et al.*[28] reported that telomerase activity and hTERT expression are attenuated by IFN-γ treatment, mediated by interferon regulatory factor-1 (IRF-1) in human cancer cell lines. As demonstrated in Figure 6, hTERT expression was decreased in LU99, LC99A and QG56-A24 cells after the IFN-γ treatment but not in PC9, 11-18 or MEG-01 cells. Taking into account the uniform up-regulation of immunoproteasome genes, TAPs and HLA-A*2402 molecules, the results strongly suggest that epitope supply to the surfaces of PC9 cells was increased and decreased to those of LU99 cells after IFN-γ treatment. Lee *et al.*[28] reported that induction of IRF-1 was closely correlated with attenuation of hTERT expression induced by IFN-γ treatment. However, IRF-1 induction was observed not only in cell lines such as LU99, LC99A and QG56-A24 where hTERT expression decreased but also in PC9 and MEG-01 where it did not (Fig. 6).

Attenuation of hTERT expression and K3-1-mediated cell lysis of primary lung cancer cells after IFN-γ treatment

Finally, we tested primary adenocarcinoma cells, obtained from a pulmonary fluid sample, to see the impact of IFN-γ treatment on hTERT expression and sensitivity to K3-1. As demonstrated in Figure 7, both hTERT expression and K3-1-medited cell lysis were attenuated after IFN-γ treatment. The results strongly suggest that IFN-γ impacts on hTERT expression and sensitivity to hTERT-specific CTLs *in vivo* as well as *in vitro*.

DISCUSSION

It was earlier reported that an HLA-A24-restricted $hTERT_{461}$-specific CTL could efficiently lyse hematological malignancies.[14] Thus, in our study, we addressed the question whether this epitope-specific CTL could similarly lyse lung cancer cells. An $hTERT_{461}$-specific CTL clone, K3-1, was generated from a healthy donor by repeated peptide stimulation and demonstrated to specifically lyse at least some lung cancer cell lines in an HLA-A24-restricted fashion. However, other examples of HLA-A24-positive lung cancer cell lines were not effectively lysed (Fig. 2*a*, center column), despite possessing telomerase activity. Sequence analysis of hTERT in these lung cancer cell lines revealed no mutation around the epitope (data not shown). Furthermore, K3-1 cytotoxicity against these cell lines was enhanced in the presence of cognate peptide, suggesting an insufficient epitope density. Pretreatment of the cell lines with IFN-γ did not, however, augment the CTL-mediated cytotoxicity. Ayyoub *et al.*[31] reported that an HLA-A2-restricted hTERT peptide 540–548-specific CD8+ T cells did not recognize tumor because of inefficient antigen processing, and we speculate that the epitope $hTERT_{461}$ is not processed and/or presented efficiently in some cell lines for unknown reasons.

IFN-γ plays important roles in the immune response not only to virus infection but also to tumors, up-regulating various genes including HLA class I,[32,33], ER peptide transporters (*e.g.* TAP1, 2),[34,35] proteasome β subunits (*e.g.* LMP2, 7, 10)[36–38] and proteasome regulators (*e.g.* PA28),[39], which contribute to antigen pro-

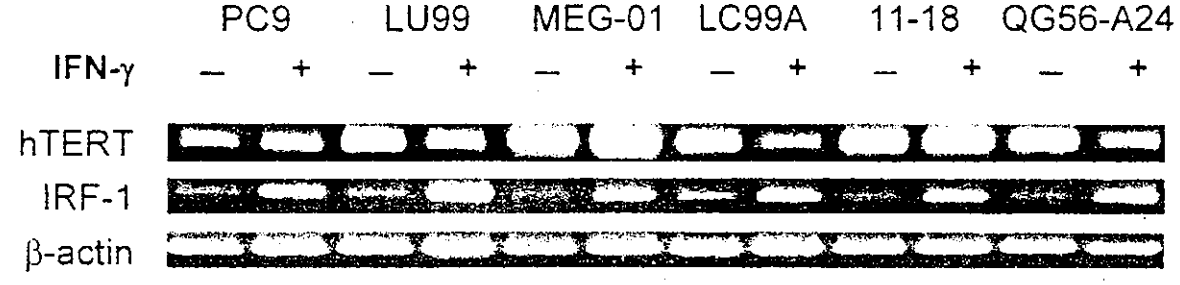


FIGURE 6 – RT-PCR analysis of hTERT and IRF-1 in cancer cell lines. Semi-quantitative RT-PCR analysis was performed using specific primers to hTERT, IRF-1 and β-actin. The mRNAs were isolated from cancer cells either treated or untreated with IFN-γ for 48 hr.

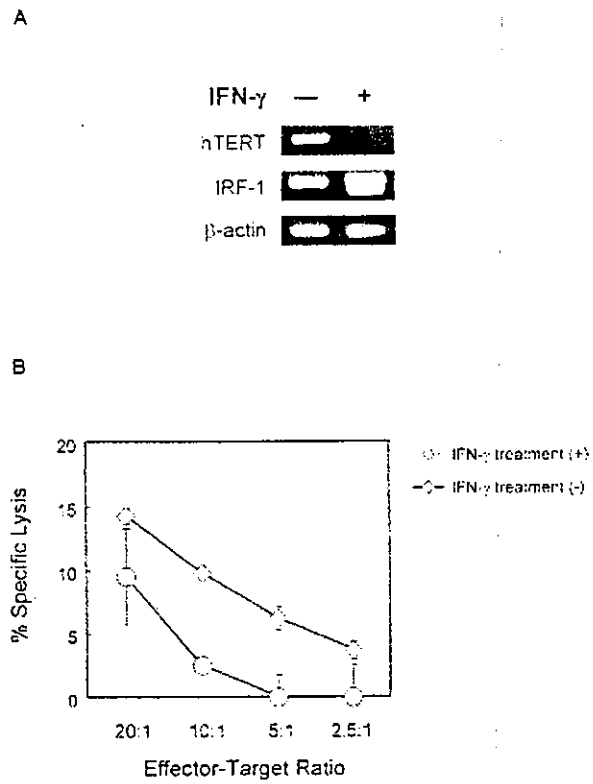


FIGURE 7 – Impact of IFN-γ treatment on primary lung cancer cells. (*a*) Freshly isolated adenocarcinoma cells from an HLA-A24-positive patient was treated or untreated with IFN-γ for 48 hr. Semi-quantitative RT-PCR analysis was performed using specific primers to hTERT, IRF-1 and β-actin. (*b*) Cytotoxicity of the $hTERT_{461}$-specific CTL clone K3-1 was determined with (open circle) or without (open diamond) IFN-γ pre-treatment (100 U/ml for 48 hr). Assays were performed at the indicated effector-target ratios. Bars indicate standard deviations.

cessing and presentation. In general, IFN-γ treatment is believed to enhance the presentation of peptides in the context of HLA class I molecules on the surface of target cells, leading to more efficient recognition by CTL. In our study, however, K3-1-mediated lysis of PC9, LU99, LC99A and MEG-01 cells was differentially affected by IFN-γ pre-treatment (Fig. 3). In addition, CTL assays using acid extracted peptides from cells indicated that the epitope was increased on PC9 but not LU99 cells after IFN-γ treatment (Fig 5*b*).

Paradoxical effects of IFN-γ on CTL recognition have in fact been reported. One example is induction of immunoproteasomes by IFN-γ treatment, with destruction of RU1-specific CTL epitopes and loss of CTL recognition.[40] In our study, immunoproteasomes did not appear to cleave the peptide within the $hTERT_{461}$ because the K3-1-mediated cytotoxicity against PC9 or MEG-01 cells was enhanced after IFN-γ treatment. In addition, LCL have been shown to express immunoproteasomes constitutively,[41] as here confirmed by Western blot analysis (Fig. 4*c*). Some telomerase-positive LCL were efficiently lysed by K3-1 in an HLA-A24-restricted fashion (data not shown), suggesting that immunoproteasomes do not destroy the $hTERT_{461}$ epitope. Another paradoxical effect of IFN-γ is mediated by inhibitory natural killer cell receptors expressed on the effector cells inducing inhibitory signaling.[42] Such receptors bind to several HLA-class I molecules, which could be upregulated by IFN-γ, thus executing the inhibitory effect of CTL-mediated target cell lysis after treatment with IFN-γ. Indeed, Malmberg *et al.*[43] reported that IFN-γ treatment inhibited lysis of ovarian cancer cells by specific CTL *via* a CD94/NKG2A-dependent mechanism. However, this could be excluded in the present case because LU99 cells treated with IFN-γ were efficiently lysed after being pulsed with cognate peptide (Fig. 3), and surface expression of CD94 on K3-1 was not detected by flow cytometric analysis (data not shown). In addition, inhibitory natural killer cell receptors, such as p58.1/KIR2DL1 or p58.2/KIR2DL2/3, were not found to be expressed on K3-1 (data not shown).

We demonstrated that hTERT expression itself was attenuated in the LU99 cells after IFN-γ treatment, resulting in inefficient recognition by the hTERT-specific CTL. The same attenuation was observed in primary lung cancer cells obtained from a lung cancer patient (Fig.7). A few reports revealed that IFN-γ reduces the expression of tumor antigens, such as MART-1/Melan A[44] or murine gp70.[45] With regard to these antigens, IFN-γ may promote immune-escape of tumors because these are not necessary for tumor proliferation. However, it is of particular interest to consider the effects of IFN-γ on telomerase activity in cancer immunity. IFN-γ may exert an anti-tumor influence primarily by suppressing hTERT transcription, resulting in limited proliferative potential. If such hTERT suppression is no longer occurring by whatever mechanism, such as IRF-1 gene inactivation as observed in some cancer cells,[46] IFN-γ might increase hTERT epitope processing and presentation leading to augmented susceptibility to specific CTL, as shown in PC9 cells (Figs. 3 and 5). Thus, the effects of IFN-γ on tumor cells through modulation of hTERT expression can be considered to feature a "fail safe" mechanism for efficient anti-tumor activity due to its impact on innate and adaptive immunity. With regard to clinical application, immunotherapy for malignant tumors using hTERT-specific CTL has unique advantages. hTERT-specific CTL not only kill tumor cells through the recognition of epitopes expressed on their surfaces but also produce and release IFN-γ *in situ*. Indeed, Le Poole *et al.*[44] reported that examination of melanoma lesions by quantitative reverse transcriptase-polymerase chain reaction revealed up to 188-fold more abundant IFN-γ transcripts produced by T cells when compared to control skin. In such circumstances, hTERT expression of tumor cells could be downregulated, resulting in suppressed tumor growth. However, some HLA-A24 positive lung cancer cells with hTERT expression were not efficiently recognized by hTERT-specific CTL, probably because of low epitope density on the cell surface. The effects of hTERT-specific CTL against such tumor cells might thus be limited. Downregulation of K3-1-mediated lysis was less pronounced with LC99A cells, although there was clear attenuation of hTERT transcription after IFN-γ treatment (Figs. 3 and 6). The reason is unclear but it could be speculated that more efficient processing and/or presentation might compensate for any shortage of hTERT proteins.

A previous study revealed that hTERT transcription may be decreased after IFN-γ treatment through induction of IRF-1.[28] Our study also confirmed downregulation of hTERT expression after IFN-γ treatment in 3 of 6 cell lines examined and primary lung cancer cells from a patient, in parallel with IRF-1 induction. However, in PC9, MEG-01 and 11–18 cells where hTERT expression did not decrease, IRF-1 was also induced. These equivocal findings for IRF-1 might be related with functional inactivation of the IRF-1 gene[46,47] or deletion or mutation of putative IRF-1 binding sites in the hTERT promoter. Alternatively, other yet-to-be identified third party molecules that cooperate with IRF-1 might be inactivated. Further studies are required to clarify the

mechanisms underlying the effect of IFN-γ upon down-regulation of hTERT expression.

In conclusion, we propose here a mechanism of attenuated CTL-mediated lysis of tumor cells through hTERT down-regulation induced by IFN-γ. Our study indicates that hTERT-specific CTL-based immunotherapy could be effective in patients with lung cancers which present relevant epitopes.

ACKNOWLEDGEMENTS

Valuable discussion and suggestions by Dr. T. Tsurumi, Dr. M. Yasukawa and Dr. T. Kiyono are highly appreciated. We are very grateful to Y Matsudaira and H Tamaki for their secretarial help. We also greatly appreciate Dr. M. Seto and Dr. T. Takahashi for generously donating cancer cell lines.

REFERENCES

1. Cross SH, Allshire RC, McKay SJ, McGill NI, Cooke HJ. Cloning of human telomeres by complementation in yeast. Nature 1989;338: 771–4.
2. Counter CM, Avilion AA, LeFeuvre CE, Stewart NG, Greider CW, Harley CB, Bacchetti S. Telomere shortening associated with chromosome instability is arrested in immortal cells which express telomerase activity. EMBO J 1992;11:1921–9.
3. Harley CB, Futcher AB, Greider CW. Telomeres shorten during ageing of human fibroblasts. Nature 1990;345:458–60.
4. Kim NW, Piatyszek MA, Prowse KR, Harley CB, West MD, Ho PL, Coviello GM, Wright WE, Weinrich SL, Shay JW. Specific association of human telomerase activity with immortal cells and cancer. Science 1994;266:2011–5.
5. Kipling D. Telomerase: immortality enzyme or oncogene? Nat Genet 1995;9:104–6.
6. Feng J, Funk WD, Wang SS, Weinrich SL, Avilion AA, Chiu CP, Adams RR, Chang E, Allsopp RC, Yu J, Le S, West MD, et al. The RNA component of human telomerase. Science 1995;269:1236–41.
7. Nakayama J, Saito M, Nakamura H, Matsuura A, Ishikawa F. TLP1: a gene encoding a protein component of mammalian telomerase is a novel member of WD repeats family. Cell 1997;88:875–84.
8. Harrington L, McPhail T, Mar V, Zhou W, Oulton R, Bass MB, Arruda I, Robinson MO. A mammalian telomerase-associated protein. Science 1997;275:973–7.
9. Meyerson M, Counter CM, Eaton EN, Ellisen LW, Steiner P, Caddle SD, Ziaugra L, Beijersbergen RL, Davidoff MJ, Liu Q, Bacchetti S, Haber DA, et al. hEST2, the putative human telomerase catalytic subunit gene, is up-regulated in tumor cells and during immortalization. Cell 1997;90:785–95.
10. Counter CM, Meyerson M, Eaton EN, Ellisen LW, Caddle SD, Haber DA, Weinberg RA. Telomerase activity is restored in human cells by ectopic expression of hTERT (hEST2), the catalytic subunit of telomerase. Oncogene 1998;16:1217–22.
11. Weinrich SL, Pruzan R, Ma L, Ouellette M, Tesmer VM, Holt SE, Bodnar AG, Lichtsteiner S, Kim NW, Trager JB, Taylor RD, Carlos R, et al. Reconstitution of human telomerase with the template RNA component hTR and the catalytic protein subunit hTRT. Nat Genet 1997;17:498–502.
12. Bodnar AG, Ouellette M, Frolkis M, Holt SE, Chiu CP, Morin GB, Harley CB, Shay JW, Lichtsteiner S, Wright WE. Extension of life-span by introduction of telomerase into normal human cells. Science 1998;279:349–52.
13. Arinaga M, Shimizu S, Gotoh K, Haruki N, Takahashi T, Mitsudomi T. Expression of human telomerase subunit genes in primary lung cancer and its clinical significance. Ann Thorac Surg 2000;70:401-5; discussion 05–6.
14. Arai J, Yasukawa M, Ohminami H, Kakimoto M, Hasegawa A, Fujita S. Identification of human telomerase reverse transcriptase-derived peptides that induce HLA-A24-restricted antileukemia cytotoxic T lymphocytes. Blood 2001;97:2903–7.
15. Vonderheide RH, Hahn WC, Schultze JL, Nadler LM. The telomerase catalytic subunit is a widely expressed tumor-associated antigen recognized by cytotoxic T lymphocytes. Immunity 1999;10:673–9.
16. Hernandez J, Garcia-Pons F, Lone YC, Firat H, Schmidt JD, Langlade-Demoyen P, Zanetti M. Identification of a human telomerase reverse transcriptase peptide of low affinity for HLA A2.1 that induces cytotoxic T lymphocytes and mediates lysis of tumor cells. Proc Natl Acad Sci U S A 2002;99:12275–80.
17. Minev B, Hipp J, Firat H, Schmidt JD, Langlade-Demoyen P, Zanetti M. Cytotoxic T cell immunity against telomerase reverse transcriptase in humans. Proc Natl Acad Sci U S A 2000;97:4796–801.
18. Nair SK, Heiser A, Boczkowski D, Majumdar A, Naoe M, Lebkowski JS, Vieweg J, Gilboa E. Induction of cytotoxic T cell responses and tumor immunity against unrelated tumors using telomerase reverse transcriptase RNA transfected dendritic cells. Nat Med 2000;6: 1011–7.
19. Kumaki F, Kawai T, Hiroi S, Shinomiya N, Ozeki Y, Ferrans VJ, Torikata C. Telomerase activity and expression of human telomerase RNA component and human telomerase reverse transcriptase in lung carcinomas. Hum Pathol 2001;32:188–95.
20. Lee SP, Tierney RJ, Thomas WA, Brooks JM, Rickinson AB. Conserved CTL epitopes within EBV latent membrane protein 2: a potential target for CTL-based tumor therapy. J Immunol 1997;158: 3325–34.
21. Schultze JL, Michalak S, Seamon MJ, Dranoff G, Jung K, Daley J, Delgado JC, Gribben JG, Nadler LM. CD40-activated human B cells: an alternative source of highly efficient antigen presenting cells to generate autologous antigen-specific T cells for adoptive immunotherapy. J Clin Invest 1997;100:2757–65.
22. Kondo E, Topp MS, Kiem HP, Obata Y, Morishima Y, Kuzushima K, Tanimoto M, Harada M, Takahashi T, Akatsuka Y. Efficient generation of antigen-specific cytotoxic T cells using retrovirally transduced CD40-activated B cells. J Immunol 2002;169:2164–71.
23. Kuzushima K, Hayashi N, Kimura H, Tsurumi T. Efficient identification of HLA-A*2402-restricted cytomegalovirus-specific CD8(+) T-cell epitopes by a computer algorithm and an enzyme-linked immunospot assay. Blood 2001;98:1872–81.
24. Akatsuka Y, Goldberg TA, Kondo E, Martin EG, Obata Y, Morishima Y, Takahashi T, Hansen JA. Efficient cloning and expression of HLA class I cDNA in human B-lymphoblastoid cell lines. Tissue Antigens 2002;59:502–11.
25. Ikeda-Moore Y, Tomiyama H, Miwa K, Oka S, Iwamoto A, Kaneko Y, Takiguchi M. Identification and characterization of multiple HLA-A24-restricted HIV-1 CTL epitopes: strong epitopes are derived from V regions of HIV-1. J Immunol 1997;159:6242–52.
26. Altman JD, Moss PA, Goulder PJ, Barouch DH, McHeyzer-Williams MG, Bell JI, McMichael AJ, Davis MM. Phenotypic analysis of antigen-specific T lymphocytes. Science 1996;274:94–6.
27. Restifo NP, Esquivel F, Kawakami Y, Yewdell JW, Mule JJ, Rosenberg SA, Bennink JR. Identification of human cancers deficient in antigen processing. J Exp Med 1993;177:265–72.
28. Lee SH, Kim JW, Lee HW, Cho YS, Oh SH, Kim YJ, Jung CH, Zhang W, Lee JH. Interferon regulatory factor-1 (IRF-1) is a mediator for interferon-gamma induced attenuation of telomerase activity and human telomerase reverse transcriptase (hTERT) expression. Oncogene 2003;22:381–91.
29. Schwarz K, van Den Broek M, Kostka S, Kraft R, Soza A, Schmidtke G, Kloetzel PM, Groettrup M. Overexpression of the proteasome subunits LMP2, LMP7, and MECL-1, but not PA28 alpha/beta, enhances the presentation of an immunodominant lymphocytic choriomeningitis virus T cell epitope. J Immunol 2000;165:768–78.
30. Bernhard H, Maeurer MJ, Jager E, Wolfel T, Schneider J, Karbach J, Seliger B, Huber C, Storkus WS, Lotze MT, Meyer zum Buschenfelde KH, Knuth A. Recognition of human renal cell carcinoma and melanoma by HLA-A2-restricted cytotoxic T lymphocytes is mediated by shared peptide epitopes and up-regulated by interferon-gamma. Scand J Immunol 1996;44:285–92.
31. Ayyoub M, Migliaccio M, Guillaume P, Lienard D, Cerottini JC, Romero P, Levy F, Speiser DE, Valmori D. Lack of tumor recognition by hTERT peptide 540-548-specific CD8(+) T cells from melanoma patients reveals inefficient antigen processing. Eur J Immunol 2001; 31:2642–51.
32. Revel M, Chebath J. Interferon-activated genes. Trends Biochem Sci 1986;11:166–70.
33. Rosa FM, Cochet MM, Fellous M. Interferon and major histocompatibility complex genes: a model to analyse eukaryotic gene regulation? Interferon 1986;7:47–87.
34. Epperson DE, Arnold D, Spies T, Cresswell P, Pober JS, Johnson DR. Cytokines increase transporter in antigen processing-1 expression more rapidly than HLA class I expression in endothelial cells. J Immunol 1992;149:3297–301.
35. Trowsdale J, Hanson I, Mockridge I, Beck S, Townsend A, Kelly A. Sequences encoded in the class II region of the MHC related to the "ABC" superfamily of transporters. Nature 1990;348:741–4.
36. Kelly A, Powis SH, Glynne R, Radley E, Beck S, Trowsdale J. Second proteasome-related gene in the human MHC class II region. Nature 1991;353:667–8.
37. Hisamatsu H, Shimbara N, Saito Y, Kristensen P, Hendil KB, Fujiwara T, Takahashi E, Tanahashi N, Tamura T, Ichihara A, Tanaka K. Newly identified pair of proteasomal subunits regulated reciprocally by interferon gamma. J Exp Med 1996;183:1807–16.
38. Nandi D, Jiang H, Monaco JJ. Identification of MECL-1 (LMP-10) as

the third IFN-gamma-inducible proteasome subunit. J Immunol 1996; 156:2361–4.
39. Realini C, Dubiel W, Pratt G, Ferrell K, Rechsteiner M. Molecular cloning and expression of a gamma-interferon-inducible activator of the multicatalytic protease. J Biol Chem 1994;269:20727–32.
40. Morel S, Levy F, Burlet-Schiltz O, Brasseur F, Probst-Kepper M, Peitrequin AL, Monsarrat B, Van Velthoven R, Cerottini JC, Boon T, Gairin JE, Van den Eynde BJ. Processing of some antigens by the standard proteasome but not by the immunoproteasome results in poor presentation by dendritic cells. Immunity 2000;12:107–17.
41. Frisan T, Levitsky V, Polack A, Masucci MG. Phenotype-dependent differences in proteasome subunit composition and cleavage specificity in B cell lines. J Immunol 1998;160:3281–9.
42. Moretta L, Biassoni R, Bottino C, Mingari MC, Moretta A. Human NK-cell receptors. Immunol Today 2000;21:420–2.
43. Malmberg KJ, Levitsky V, Norell H, de Matos CT, Carlsten M, Schedvins K, Rabbani H, Moretta A, Soderstrom K, Levitskaya J, Kiessling R. IFN-gamma protects short-term ovarian carcinoma cell lines from CTL lysis via a CD94/NKG2A-dependent mechanism. J Clin Invest 2002;110:1515–23.
44. Le Poole IC, Riker AI, Quevedo ME, Stennett LS, Wang E, Marincola FM, Kast WM, Robinson JK, Nickoloff BJ. Interferon-gamma reduces melanosomal antigen expression and recognition of melanoma cells by cytotoxic T cells. Am J Pathol 2002;160:521–8.
45. Beatty GL, Paterson Y. IFN-gamma can promote tumor evasion of the immune system in vivo by down-regulating cellular levels of an endogenous tumor antigen. J Immunol 2000;165:5502–8.
46. Willman CL, Sever CE, Pallavicini MG, Harada H, Tanaka N, Slovak ML, Yamamoto H, Harada K, Meeker TC, List AF, Taniguchi T. Deletion of IRF-1, mapping to chromosome 5q31.1, in human leukemia and preleukemic myelodysplasia. Science 1993;259:968–71.
47. Nozawa H, Oda E, Ueda S, Tamura G, Maesawa C, Muto T, Taniguchi T, Tanaka N. Functionally inactivating point mutation in the tumor-suppressor IRF-1 gene identified in human gastric cancer. Int J Cancer 1998;77:522–7.

REVIEW

The impact of *EGFR* mutations on gefitinib sensitivity in non-small cell lung cancer

Takeshi Suzuki[1], *Tetsuya Mitsudomi*[2] & *Toyoaki Hida*[†3]

[†]Author for correspondence
[1]Department of Internal Medicine and Molecular Science, Nagoya City University Medical School, Mizuho-ku, Nagoya 467-8601, Japan
[2]Department of Thoracic Surgery, Aichi Cancer Center Hospital, 1-1 Kanokoden, Chikusa-Ku, Nagoya 464-8681, Japan
[3]Department of Thoracic Oncology, Aichi Cancer Center Hospital, 1-1 Kanokoden, Chikusa-Ku, Nagoya 464-8681, Japan
Fax: +81 52 763 5233;
E-mail: 107974@aichi-cc.jp

Gefitinib (ZD1839, Iressa®; AstraZeneca) has produced objective tumor responses and symptom improvement in some patients with non-small cell lung cancer. In clinical trials, 12–18.4% of patients had a rapid and often dramatic clinical response, and a subset analysis of the Iressa Dose Evaluation in Advanced Lung Cancer (IDEAL)-1 and -2 trials demonstrated that female gender and adenocarcinoma were associated with a higher response to gefitinib. However, analysis from clinical trials have not found a relationship between epidermal growth factor receptor (EGFR) expression and response in patients receiving gefitinib. Recently, three studies have identified mutations affecting the EGFR in lung cancer from patients who respond to gefitinib. *EGFR* gene mutations were common in lung cancer from 'never smokers' and were associated with sensitivity of tumors to gefitinib. Furthermore, it has been reported that the phosphatidylinositol 3-kinase/Akt signaling pathway plays a critical role in the antitumor effects of gefitinib. Although *EGFR* mutations do not fully explain the clinical benefit, the data regarding *EGFR* mutations may help to define the patient population that will most likely benefit from EGFR tyrosine-kinase-targeted therapies.

The epidermal growth factor receptor (EGFR, also known as ErbB1 or HER1) is a transmembrane receptor tyrosine kinase (TK) of the HER family (HER2/neu [ErbB2], HER3 [ErbB3], and HER4 [ErbB4]), and is activated by the extracellular binding of a variety of ligands [1]. The receptor plays an important role in the proliferation and metastasis of tumor cells [1], and several human cancers (e.g., cancers of the upper aerodigestive tract, colon, pancreas, breast, ovary, bladder, kidney, and gliomas) display EGFR RNA and/or protein overexpression [2-10]. Current molecularly targeted agents aim at inhibiting specific pathways and key molecules that are involved in tumor growth and progression, while sparing normal cells. Examples of such agents include trastuzumab, which is a monoclonal antibody that targets the HER2/neu receptor protein in breast cancer, and imatinib mesylate, a small molecule receptor tyrosine kinase inhibitor (TKI) that targets Bcr/abl and c-Kit in chronic myelogenous leukemia and gastrointestinal stromal tumors, respectively [11,12]. Depending on the specific molecule targeted and the mechanism of inhibition, these agents may offer novel clinical benefits compared with the outcomes of cytotoxic chemotherapy, or at least comparable benefits with reduced general toxicity and improved convenience.

The TK activity of EGFR has received considerable attention as a target for cancer therapy [1,13-15]. In recent clinical trials, the selective and orally active EGFR TKI gefitinib (ZD1839, Iressa®; AstraZeneca) has produced objective tumor responses and symptom improvement in some patients with non-small cell lung cancer (NSCLC) who had previously received chemotherapy [16,17]. Partial clinical responses to gefitinib have been observed most frequently in females, non-smokers and patients with adenocarcinomas [16-18]. In addition, a significant variability in the response to gefitinib has been noted, with higher responses seen in Japanese patients when compared with a predominantly European-derived population (27.5 versus 10.4%) [16]. The EGFR TKIs are the first class of oral targeted therapies to produce such responses in advanced NSCLC. However, two large, multinational Phase III studies of gefitinib in combination with standard platinum-based chemotherapy regimens in the first-line treatment of patients with NSCLC did not show any added advantage [19,20].

The molecular mechanisms underlying sensitivity to gefitinib were unknown. However, three recent studies have identified mutations affecting the EGFR present in lung cancer tissues from patients who responded to gefitinib. These findings may help select patients who can benefit from treatment with gefitinib in the future. This review will concentrate on the agent most advanced in clinical development, the EGFR TKI gefitinib, and discuss the predictive factors for response to gefitinib.

Keywords: Akt, EGFR, *EGFR* mutation, epidermal growth factor receptor, gefitinib, gefitinib sensitivity, non-small cell lung cancer, NSCLC, tyrosine kinase inhibitor

future medicine

Gefitinib

EGFR is a ubiquitous 170-kDa membrane-spanning glycoprotein composed of an amino-terminal extracellular ligand-binding domain; a hydrophobic transmembrane region; a cytoplasmic domain that contains the TK domain; and a carboxy-terminal region that contains critical tyrosine residues and receptor regulatory motifs [21]. Ligand binding leads to the formation of a variety of activated ErbB receptor homo- and heterodimer complexes. Dimerization produces structural changes in the intracellular portion of the receptor that activate the TK domain. The enzymatic activity of EGFR TK transfers phosphate moieties from ATP to specific tyrosine residues in the cytoplasmic tail of the EGFR protein. These phosphorylated tyrosines serve as the docking sites for a number of signal transducers and adaptor molecules that initiate a plethora of signaling pathways, resulting in cell proliferation, differentiation, migration, adhesion, protection from apoptosis, and transformation (Figure 1).

Among the multiple signal transduction pathways activated by the EGF family, the mitogen-activated protein kinase (MAPK; ERK-2) pathway is one of the most relevant; it regulates cellular processes, such as gene transcription and proliferation, by activating a variety of substrates located in the cytosol, nucleus, and plasma membrane [22-24]. Another important signal transduction pathway activated by the EGF family of receptors is the phosphatidylinositol 3-kinase (PI3K)/Akt signaling pathway, which mediates cell survival [25,26].

Different approaches to inhibiting EGFR have resulted in a number of EGFR-targeted agents in clinical development, including small molecule EGFR TKIs, monoclonal antibodies, vaccines, immunotoxins, and recombinant ligand–toxin fusion proteins. Small molecule EGFR TKIs act by blocking the ATP-binding site of the EGFR TK enzyme inside tumor cells. Based on this mechanism of action, EGFR TKIs have the potential to inhibit all mechanisms of EGFR TK activation, including constitutively activating mutations and receptor crosstalk. EGFR TKIs were designed to selectively inhibit EGFR TK relative to other kinase enzymes present in normal tissues [27].

Gefitinib is a low-molecular-weight (447 kDa) quinazoline derivative that specifically inhibits the activation of EGFR TK through the competitive binding of the ATP-binding domain of the receptor. The selectivity of the compound for EGFR was demonstrated against HER2 TK and the two vascular endothelial growth factor (VEGF) receptors KDR TK and Flt-1 TK, and a $\geq$ 100-fold difference in IC_{50} was noted for EGFR compared with the other TKs. Similarly, gefitinib did not inhibit the activity of the serine threonine kinases raf, MEK-1, and MAPK [28]. Compared with anti-EGFR monoclonal antibodies, such as cetuximab, gefitinib offers the advantages of oral bioavailability and once-daily treatment. The drug was effective in preclinical models for inhibiting the growth of a variety of human tumor cell lines, including lung, colorectal, oral, and prostate, suggesting the potential for its broad applicability in the treatment of solid tumor types [29]. Furthermore, lung tumor xenografts have been inhibited by gefitinib alone or in combination with chemotherapy agents [29]. Gefitinib has been shown to potentiate the antitumor effects of most cytotoxic agents, including platinum-based chemotherapy agents in preclinical models with cell lines sensitive to EGFR inhibition [29,30]. In addition, gefitinib has shown activity against NSCLC xenografts in combination with taxanes, doxorubicin, and antifolates [29,30].

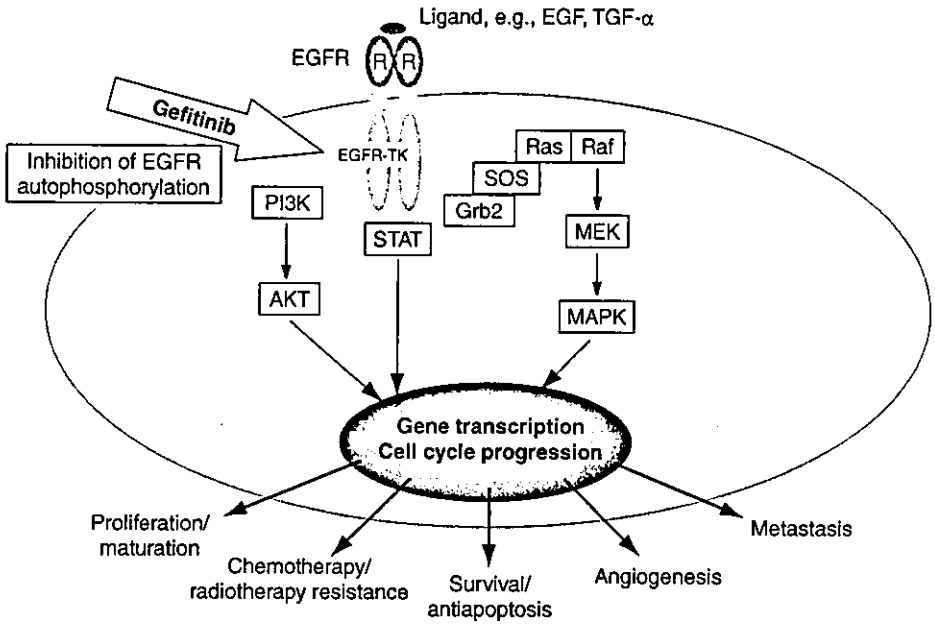
Pharmacodynamic studies indicate that gefitinib blocks cell cycle progression in the G1 phase by upregulating $p27^{Kip1}$, a cell cycle inhibitor, and downregulating c-fos, a transcriptional activator that is prominent in EGFR-mediated signaling [31]. Elevated levels of $p27^{Kip1}$ block cell cycle progression in the G1 phase of growth, thus sustaining the hypophosphorylated state of the retinoblastoma (*RB1*) gene product, which is necessary to keep cells from progressing in the cell cycle [32]. The inhibition of tumor growth seen with gefitinib is also accompanied by decreases in VEGF, basic fibroblast growth factor (bFGF), and TGF-α, which are all potent inducers of tumor angiogenesis [30]. Thus, gefitinib may also inhibit tumor growth by interfering with angiogenesis.

These observations suggest that by inhibiting the EGFR TK, gefitinib treatment alters the expression levels of key molecules in tumor cells that are important for stimulating proliferation, cell cycle progression, tumor angiogenesis, metastasis, and inhibition of apoptosis.

EGFR gene mutations

Recently, three clinical studies have identified mutations affecting the EGFR in lung cancers from patients who respond to gefitinib. These reports identified somatic mutations in the

Figure 1. The mechanism of action of gefitinib signal transduction blockage through EGFR tyrosine kinase.



EGF: Epidermal growth factor; EGFR: Epidermal growth factor receptor; Grb2: Growth factor receptor-binding protein 2; MAPK: Mitogen-activated protein kinase; PI3K: Phosphatidylinositol 3-kinase; R: Receptor; STAT: Signal transducers and activators of transcription; TGF: Transforming growth factor; TK: Tyrosine kinase.

EGFR gene in NSCLC patients that showed a clinical response to gefitinib. The mutations were short, in-frame deletions or substitutions clustered around the region encoding the ATP-binding pocket of the receptor's TK domain.

Lynch *et al.* [33] used primary lung tumor samples from responders to gefitinib versus non-responders to search for mutations in *EGFR*. They found that heterozygous gain-of-function mutations were clustered within the TK domain of the EGFR (the active site where gefitinib binds) in tumor specimens analyzed from eight of nine patients who were sensitive to gefitinib. Of these patients, four had in-frame deletions within exon 19 and three had amino acid substitutions within exon 21. Matched normal tissue from these patients did not contain the mutations and no mutations were seen in seven patients who had not responded to gefitinib. Paez *et al.* [34] screened Japanese and American patients with NSCLC for mutations restricted to the activation loop (A-loop) of a large subset of all human membrane receptor TKs. They also examined samples from patients treated with gefitinib for mutations in *EGFR* and found mutations similar to those in the study by Lynch *et al* [33].

Both papers showed a correlation of the mutations with certain patient characteristics, which, in turn, correlated with the subset of patients who respond to gefitinib. In the study by Paez *et al.* [34], these mutations were more frequent in female and Japanese patients, as well as in patients with adenocarcinoma compared with other histological types of NSCLC. This report showed that 15 (29%) out of the 58 Japanese patients were heterozygous for the *EGFR* mutations in their tumor tissue but not in their normal tissue, compared with only 1 patient out of 61 from the USA. The result corresponds to the findings of the first Iressa Dose Evaluation in Advanced Lung Cancer (IDEAL-1) trial, which showed a 27.5% response rate in Japanese patients [16]. An association was also seen in both studies between the presence of mutations and gefitinib sensitivity in female patients with bronchioloalveolar carcinoma (a subtype of adenocarcinoma) who were not current smokers, further confirming the subset of patients known to respond to gefitinib. In addition, Pao *et al.* [35] showed that adenocarcinomas from 'never smokers' (patients who had smoked < 100 cigarettes in their lifetime) comprised a distinct subset of lung cancers, frequently containing mutations within the TK domain of

EGFR that were associated with sensitivity to gefitinib and erlotinib (OSI-774, Tarceva®; another selective EGFR TKI).

On the genomic level, sequence data presented by both groups [33,34] showed that point mutations in exons 18 and 21, respectively, are heterozygous. This contrasts with deletions in exon 19 where, in some of the sequencing traces presented, the normal allele is severely under-represented or absent, indicating a loss of heterozygosity or amplification of the mutant locus. Thus, the point mutations in exons 18 and 21 may be dominant in heterodimers consisting of a normal and a mutant EGFR or an EGFR family member. In contrast, mutations in exon 19 may be functionally recessive and possibly require homodimerization for phenotypic penetrance [36].

Lynch *et al.* went on to show that transfection of the missense EGFR mutant Leu858Arg or the in-frame deletion Leu747–Pro753insSer into cultured cell lines resulted in a two- to threefold increased stimulation of the receptor by EGF and that activation of the mutant receptor lasted up to 12-times longer when compared with the wild-type receptor [33]. Furthermore, the mutant EGFR proteins were approximately 10 times more sensitive to inhibition by gefitinib than the wild-type EGFR. Paez *et al.* investigated a cell line derived from a malignant pleural effusion from a Caucasian female non-smoker with lung adenocarcinoma [34]. The adenocarcinoma had the Leu858Arg mutation in EGFR and was 50 times more sensitive to gefitinib than other adenocarcinoma cell lines. The authors hypothesized that the mutations in EGFR stabilize the interaction between the drug and the kinase, thereby increasing the inhibitory effect of gefitinib. If the association between the *EGFR* mutations and gefitinib sensitivity are confirmed in prospective clinical trials, they will set a standard for approaches to the evaluation and use of targeted therapy for solid tumors.

In the Aichi Cancer Center in Japan, Kosaka *et al.* found that ~ 40% of NSCLC Japanese patients who had undergone resection carried *EGFR* mutations (*Cancer Research*, in press). In their study, RNA was extracted and direct sequencing of the TK domain of the *EGFR* gene was performed after reverse transcription-PCR. The mutations were mostly deletions around codons 746–750 or point mutations, predominantly at codon 858; rarer mutations were also noted. *EGFR* mutations were significantly frequent in females, patients with adenocarcinomas and patients who had never smoked. They also found that *EGFR* mutations and K-*ras* mutations were mutually exclusive, whereas *EGFR* mutations were independent of p53 (*TP53*) mutations. They also studied the patients who were treated with gefitinib for recurrent diseases after surgery. *EGFR* mutations showed good but not perfect correlation with gefitinib effectiveness, even after prior chemotherapy treatment (before the application of gefitinib), as reported previously (**Table 1**). Patients with *EGFR* mutations survived for a longer period than those without the mutations after initiation of gefitinib treatment (Mitsudomi T *et al.*, submitted for publication).

Tumor mutations that are insensitive to kinase inhibitors, such as those found in human glioblastomas, include extensive deletions or missense mutations in the extracellular domain of the EGFR, or deletions of the regulatory intracellular domain (but not the kinase domain). In contrast, many NSCLCs carrying missense mutations and deletions in *EGFR* are sensitive to kinase inhibitors, such as gefitinib. These mutations are found in three distinct sites in the EGFR kinase domain: the phosphate-binding loop (P-loop), the A-loop, and the αC helix.

It is not clear from these studies whether only tumors with the aforementioned *EGFR* mutations respond to gefitinib. In the Paez *et al.* study [34], 1 out of 61 patients from the USA had *EGFR* mutations in their tumor tissue, which is far lower than the 10% response rate reported by Kris *et al.* in the Phase II study of gefitinib in the USA [17]. In the study by Lynch *et al.* [33], one of nine responding patients had a wild-type receptor. In previous gefitinib studies, there was a group of patients that showed marked symptom improvement and prolonged disease stabilization with no measurable reduction in tumor size. However, the *EGFR* status of such tumors was not reported in either study.

Other molecular correlates of gefitinib response

Akt and STAT phosphorylation

Cappuzzo *et al.* [40] evaluated the two major EGFR signaling pathways (PI3K/Akt and Ras/Raf/MAPK) immunohistochemically, and found that only the PI3K/Akt pathway was significantly associated with gefitinib activity. In their study, Akt was activated in ~ 50% of patients with NSCLC. Furthermore, phosphorylation of Akt (P-Akt) positivity was associated with being female, with never smoking, and with

Table 1. *EGFR* gene mutations and response to gefitinib.

Study	No. of patients with mutations/no. of patients with clinical response	No. of patients with mutations/no. of patients with no clinical response
Paez *et al.* [34], Jänne *et al.* [37]	100% (7/7)	0% (0/6)
Lynch *et al.* [33,38]	89% (8/9)	0% (0/7)
Cappuzzo *et al.* [39]	89% (8/9)	33% (3/9)
Pao *et al.* [35]	70% (7/10)	0% (0/8)

EGFR: Epidermal growth factor receptor.

bronchioloalveolar carcinoma histology. Patients with P-Akt-positive tumors who received gefitinib had a better response rate, disease control rate and time to progression than patients with P-Akt-negative tumors, suggesting that gefitinib may be more effective in patients with basal Akt activation. In the multivariable analysis, P-Akt status was significantly associated with a reduced risk of disease progression (hazard ratio [HR] = 0.58, 95% confidence interval [CI] 0.35–0.94). Importantly, after being adjusted for P-Akt status, performance status and smoking history remained significantly associated with an increased risk of disease progression (HR = 2.65 [95% CI 1.33–5.27] and 1.75 [95% CI 1.08–2.85], respectively), and female gender was immediately removed at the first step of the backward elimination. Recent data indicate that sensitivity to gefitinib therapy requires intact EGFR-stimulated Akt signaling activity and that loss of PTEN (phosphatase and tensin homolog; a phosphatase that negatively regulates Akt by dephosphorylating it) can lead to aberrant Akt activation and, finally, to gefitinib resistance [41-43].

Recently, Sordella *et al.* [44] reported that mutant EGFRs selectively activated Akt and STAT signaling pathways, which promote cell survival, but have no effect on Erk/MAPK signaling, which induces proliferation. EGF-induced phosphorylation of tyrosine residues at codon 1045 (Tyr1045) and Tyr1173 was virtually indistinguishable between the wild-type and mutant EGFRs, whereas phosphorylation of Tyr992 and Tyr1068 was substantially increased in the mutant EGFRs. The increased Akt and STAT phosphorylation following activation of the mutant EGFRs was consistent with the increase in Tyr992 and Tyr1068 phosphorylation. The selective EGF-induced autophosphorylation of C-terminal tyrosine residues within EGFR mutants was well correlated with the selective activation of downstream signaling pathways. Interestingly, Sordella *et al.* suggested that the expression of mutant EGFRs appeared essential for suppression of proapoptotic signals in lung cancers harboring these mutations. Sensitivity to gefitinib may result in large part from its effective inhibition of essential antiapoptotic signals transduced by the mutant receptor in lung cancer cells with EGFR kinase mutations. Thus, mutant EGFRs selectively transduce survival signals on which NSCLCs become dependent, and the inhibition of those signals by gefitinib may contribute to the efficacy of the drug.

EGFR expression

Despite promising clinical results in the treatment of NSCLC, the potential relationship of the sensitivity to gefitinib with various characteristics of NSCLC, such as EGFR expression level, is not clear. In clinical trials, objective responses were observed in lung cancer patients with adenocarcinoma that was less likely to express EGFR than squamous cell carcinoma. In a retrospective analysis from IDEAL, EGFR expression (as detected by immunohistochemistry) was not related to response in patients receiving gefitinib monotherapy [45]. Similarly, in the Phase II study with erlotinib, the intensity of HER1/EGFR staining was not associated with the clinical outcome of metastatic squamous cell cancer in head and neck patients [46]. In this regard, Sirotnak *et al.* demonstrated that the degree of potentiation of gefitinib in a variety of tumor xenografts was not dependent on high levels of expression of EGFR [29]. Using a panel of 19 lung cancer cell lines, Suzuki *et al.* observed the lack of association of gefitinib sensitivity with the expression of EGFR, HER2, HER3, and HER4 [47]. Their results also showed no apparent association between K-*ras* mutations

Highlights

- Gefitinib inhibits the growth of some lung cancers, although this effect is not well correlated with the level of expression of EGFR or related members of the ErbB family of receptors.
- Recently, three studies have identified mutations affecting EGFR in lung cancers from patients. These reports identify somatic mutations in the *EGFR* gene in patients with NSCLC that had clinical responses to the EGFR TKI gefitinib. Another study suggested that the expression of mutant EGFRs appeared essential for the suppression of proapoptotic signals in lung cancers harboring these mutations. Sensitivity to gefitinib may largely result from its effective inhibition of essential antiapoptotic signals transduced by the mutant receptor in lung cancer cells with EGFR kinase mutations. It is, however, not clear from these studies whether only tumors with *EGFR* mutations respond to gefitinib.
- If these data are confirmed in prospective clinical trials, they will set a standard for approaches to the evaluation and use of targeted therapy for solid tumors.

and sensitivity to gefitinib. Although it could be hypothesized that EGFR expression is a prerequisite for a response to the drug, EGFR expression has not turned out to be useful in the selection of patients who respond to gefitinib.

It was reported that the specificity and potency of the signaling output from activated EGFR is highly dependent on the identity of the activating ligand, as well as on the cellular levels of the coreceptors HER2, HER3, and HER4, all of which can oligomerize with EGFR. The sites autophosphorylated in the C-terminal portion of EGFR, as well as the signaling molecules that associate with the receptor, are determined by the heterodimeric partner of EGFR. Thus, other members of the HER network may influence the efficacy of gefitinib.

Gene expression profiling and proteomic analysis

In a microarray analysis, Hirsch *et al.* [48] identified 13 genes for which expression accurately discriminated between gefitinib-sensitive and -resistant NSCLC cell lines. Carbone *et al.* [49] presented results of a proteomic analysis of fresh tumor samples from NSCLC patients resistant or sensitive to gefitinib treatment. This analysis identified six protein peaks that were 95% predictive of response to gefitinib and 88 peaks that were 100% predictive. These observations require prospective validation in large studies.

Outlook

In clinical studies with NSCLC patients, gefitinib has shown modest clinical activity, with higher response rates in females, patients with lung adenocarcinoma, non-smokers, and patients of Japanese origin. In none of these trials were patients selected based on evidence of EGFR kinase dependence. Three studies by Paez *et al.* [34], Lynch *et al.* [33] and Pao *et al.* [35] have revealed some of the molecular underpinnings of the overall low clinical activity of EGFR inhibitors and will almost certainly lead to the identification of subgroups of patients who are likely to benefit substantially from these drugs. It is, however, not clear from these studies whether only tumors with *EGFR* mutations respond to gefitinib. The clear message from studies of *EGFR* mutations is that although these mutations are important in determining a dramatic response to EGFR TKIs, this is not the whole story. In addition, whether these mutant receptors depend on ligands for activation *in situ* is a crucial question. Ligand-independent activation could anticipate therapeutic resistance to the ligand-blocking EGFR antibodies that are currently in clinical development.

It is clear from these studies that molecular target dependence and patient selection should be central to the development of molecular therapeutics in human cancer. This approach should avoid the spuriously negative or overall weak signals of clinical activity of drugs that are otherwise very active when used in the right group of patients; prevent unnecessary large, costly trials; and limit the exposure of patients to drugs unlikely to produce any clinical benefit. In EGFR-mutant lung cancers that eventually escape gefitinib treatment, it is now important to determine whether the resistance is EGFR-dependent or -independent.

Bibliography

Papers of special note have been highlighted as either of interest (•) or of considerable interest (••) to readers.

1. Mendelson J: Blockade of receptors for growth factors: an anticancer therapy – the Fourth Annual Joseph H Burchenal American Association of Cancer Research Clinical Research Award Lecture. *Clin. Cancer Res.* 6(3), 747-753 (2000).
2. Salomon DS, Brandt R, Ciardiello F *et al.*: Epidermal growth factor-related peptides and their receptors in human malignancies. *Crit. Rev. Oncol. Hematol.* 19(3), 183-232 (1995).
3. Rusch V, Baselga J, Cordon-Cardo C *et al.*: Differential expression of the epidermal growth factor receptor and its ligands in primary non-small cell lung cancers and adjacent benign lung. *Cancer Res.* 53(10, Suppl.), 2379-2385 (1993).
4. Klijn JG, Berns PM, Schmitz PI *et al.*: The clinical significance of epidermal growth factor receptor (EGF-R) in human breast cancer: a review on 5232 patients. *Endocr. Rev.* 13(1), 3-17 (1992).
5. Rubin Grandis J, Melhem MF, Gooding WE *et al.*: Levels of TGF-α and EGFR protein in head and neck squamous cell carcinoma and patient survival. *J. Natl. Cancer Inst.* 90(11), 824-832 (1998).
6. Yonemura Y, Takamura H, Ninomiya I *et al.*: Interrelationship between transforming growth factor-α and epidermal growth factor receptor in advanced gastric cancer. *Oncology* 49(2), 157-161 (1992).
7. Hirai T, Kuwahara M, Yoshida K *et al.*: Clinical results of transhiatal esophagectomy for carcinoma of the lower thoracic esophagus according to biological markers. *Dis. Esophagus* 11(4), 221-225 (1998).
8. Tateishi M, Ishida T, Mitsudomi T *et al.*: Immunohistochemical evidence of autocrine growth factors in adenocarcinoma of the human lung. *Cancer Res.* 50(21), 7077-7080 (1990).
9. Yamanaka Y, Friess H, Kobrin MS *et al.*: Coexpression of epidermal growth factor receptor and ligands in human pancreatic cancer is associated with enhanced tumor aggressiveness. *Anticancer Res.* 13(3), 565-569 (1993).
10. Ekstrand AJ, James CD, Cavenee WK *et al.*: Genes for epidermal growth factor receptor, transforming growth factor α, and epidermal growth factor and their expression in human gliomas *in vivo*. *Cancer Res.* 51(8), 2164-2172 (1991).
11. Vogel CL, Cobleigh MA, Tripathy D *et al.*: Efficacy and safety of trastuzumab as a single agent in first-line treatment of HER2-overexpressing metastatic breast cancer. *J. Clin. Oncol.* 20(3), 719-726 (2002).
12. Savage DG, Antman KH: Imatinib mesylate – a new oral targeted therapy. *N. Engl. J. Med.* 346(9), 683-693 (2002).
13. Hirsch FR, Varella-Garcia M, Bunn PA Jr *et al.*: Epidermal growth factor receptor in non-small-cell lung carcinomas: correlation between gene copy number and protein expression and impact on prognosis. *J. Clin. Oncol.* 21(20), 3798-3807 (2003).
14. Baselga J, Rischin D, Ranson M *et al.*: Phase I safety, pharmacokinetic, and pharmacodynamic trial of ZD1839, a selective oral epidermal growth factor receptor tyrosine kinase inhibitor, in patients with five selected solid tumor types. *J. Clin. Oncol.* 20(21), 4292-4302 (2002).
15. Herbst RS, Maddox AM, Rothenberg ML *et al.*: Selective oral epidermal growth factor receptor tyrosine kinase inhibitor ZD1839 is generally well-tolerated and has activity in non-small-cell lung cancer and other solid tumors: results of a Phase I trial. *J. Clin. Oncol.* 20(18), 3815-3825 (2002).
16. Fukuoka M, Yano S, Giaccone G *et al.*: Multi-institutional randomized Phase II trial of gefitinib for previously treated patients with advanced non-small-cell lung cancer. *J. Clin. Oncol.* 21(12), 2237-2246 (2003).
17. Kris MG, Natale RB, Herbst RS *et al.*: Efficacy of gefitinib, an inhibitor of the epidermal growth factor receptor tyrosine kinase, in symptomatic patients with non-small cell lung cancer: a randomized trial. *JAMA* 290(16), 2149-2158 (2003).
18. Miller VA, Kris MG, Shah N *et al.*: Bronchioloalveolar pathologic subtype and smoking history predict sensitivity to gefitinib in advanced non-small-cell lung cancer. *J. Clin. Oncol.* 22(6), 1103-1109 (2004).
19. Giaccone G, Herbst RS, Manegold C *et al.*: Gefitinib in combination with gemcitabine and cisplatin in advanced non-small-cell lung cancer: a Phase III trial-INTACT 1. *J. Clin. Oncol.* 22(5), 777-784 (2004).
20. Herbst RS, Giaccone G, Schiller JH *et al.*: Gefitinib in combination with paclitaxel and carboplatin in advanced non-small-cell lung cancer: a Phase III trial-INTACT 2. *J. Clin. Oncol.* 22(5), 785-794 (2004).
21. Schlessinger J: Cell signaling by receptor tyrosine kinases. *Cell* 103(2), 211-225 (2000).
22. Prenzel N, Fischer OM, Streit S *et al.*: The epidermal growth factor receptor family as a central element for cellular signal transduction and diversification. *Endocr. Relat. Cancer* 8(1), 11-31 (2001).
23. Davis RJ: Signal transduction by the JNK group of MAP kinases. *Cell* 103(2), 239-252 (2000).
24. Ballif BA, Blenis J: Molecular mechanisms mediating mammalian mitogen-activated protein kinase (MAPK) kinase (MEK)-MAPK cell survival signals. *Cell Growth Differ.* 12(8), 397-408 (2001).
25. Datta SR, Brunet A, Greenberg ME: Cellular survival: a play in three Akts. *Genes Dev.* 13(22), 2905-2927 (1999).
26. Kapeller R, Cantley LC: Phosphatidylinositol 3-kinase. *Bioessays* 16(8), 565-576 (1994).
27. Arteaga CL: The epidermal growth factor receptor: from mutant oncogene in nonhuman cancers to therapeutic target in human neoplasia. *J. Clin. Oncol.* 19(18, Suppl.), 32S-40S (2001).
28. Wakeling AE, Guy SP, Woodburn JR *et al.*: ZD1839 (Iressa): an orally active inhibitor of epidermal growth factor signaling with potential for cancer therapy. *Cancer Res.* 62(20), 5749-5754 (2002).
29. Sirotnak FM, Zakowski MF, Miller VA *et al.*: Efficacy of cytotoxic agents against human tumor xenografts is markedly enhanced by coadministration of ZD1839 (Iressa), an inhibitor of EGFR tyrosine kinase. *Clin. Cancer Res.* 6(12), 4885-4892 (2000).
30. Ciardiello F, Caputo R, Bianco R *et al.*: Inhibition of growth factor production and angiogenesis in human cancer cells by ZD1839 (Iressa), a selective epidermal growth factor receptor tyrosine kinase inhibitor. *Clin. Cancer Res.* 7(5), 1459-1465 (2001).
31. Di Gennaro E, Barbarino M, Bruzzese F *et al.*: Critical role of both $p27^{KIP1}$ and $p21^{CIP1/WAF1}$ in the antiproliferative effect of ZD1839 ('Iressa'), an epidermal growth factor receptor tyrosine kinase inhibitor, in head and neck squamous carcinoma cells. *J. Cell Physiol.* 195(1), 139-150 (2003).
32. Mendelsohn J, Fan Z: Epidermal growth factor receptor family and chemosensitization. *J. Natl. Cancer Inst.* 89(5), 341-343 (1997).
33. Lynch TJ, Bell DW, Sordella R *et al.*: Activating mutations in the epidermal growth factor receptor underlying responsiveness of non-small-cell lung cancer

to gefitinib. *N. Engl. J. Med.* 350(21), 2129-2139 (2004).

•• Showed that specific missense and deletion mutations in the TK domain of the *EGFR* gene are significantly associated with gefitinib sensitivity. Lynch *et al.* provided suggestive evidence that the mutant receptors were hyper-responsive to the exogenous EGFR ligand.

34. Paez JG, Janne PA, Lee JC *et al.*: EGFR mutations in lung cancer: correlation with clinical response to gefitinib therapy. *Science* 304, 1497-1500 (2004).

•• Showed that specific missense and deletion mutations in the TK domain of the *EGFR* gene are significantly associated with gefitinib sensitivity. Paez *et al.* screened Japanese and American NSCLC patients for mutations.

35. Pao W, Miller V, Zakowski M *et al.*: EGF receptor gene mutations are common in lung cancers from "never smokers" and are associated with sensitivity of tumors to gefitinib and erlotinib. *Proc. Natl. Acad. Sci. USA* 101, 13306-13311 (2004).

•• Showed that adenocarcinomas from never smokers comprised a distinct subset of lung cancers, frequently containing mutations within the TK domain of EGFR that were associated with gefitinib and erlotinib sensitivity.

36. Minna JD, Gazdar AF, Sprang SR *et al.*: A bull's eye for targeted lung cancer therapy. *Science* 304, 1458-1461 (2004).

37. Jänne PA: Mutations in *EGFR* and response to gefitinib and erlotinib. *40th Annual Meeting of ASCO*, New Orleans, USA (2004)

38. Lynch TJ: EGFR activating mutations underlying the responsiveness of NSCLC to gefitinib. *40th Annual Meeting of ASCO*, New Orleans, USA (2004).

39. Cappuzzo F, Magrini E, Bartolini S *et al.*: Improved efficacy of gefitinib therapy in phospho-Akt positive patients with advanced non-small cell lung cancer. *Proc. Am. Soc. Clin. Oncol.* 23, 196 (2004) (Abstract 3004).

40. Cappuzzo F, Magrini E, Ceresoli GL *et al.*: Akt phosphorylation and gefitinib efficacy in patients with advanced non-small-cell lung cancer. *J. Natl. Cancer Inst.* 96, 1133-1141 (2004).

• Showed that patienta with P-Akt-positive tumors who received gefitinib had a better response rate and disease control rate than patients with P-Akt-negative tumors.

41. She QB, Solit D, Basso A *et al.*: Resistance to gefitinib in PTEN-null HER-overexpressing tumor cells can be overcome through restoration of PTEN function or pharmacologic modulation of constitutive phosphatidylinositol 3-kinase/Akt pathway signaling. *Clin. Cancer Res.* 9, 4340- 4346 (2003).

42. Bianco R, Shin I, Ritter CA *et al.*: Loss of PTEN/MMAC1/TEP in EGF receptor-expressing tumor cells counteracts the antitumor action of EGFR tyrosine kinase inhibitors. *Oncogene* 22, 2812-2822 (2003).

43. Janmaat ML, Kruyt FA, Rodriguez JA *et al.*: Response to epidermal growth factor receptor inhibitors in non-small cell lung cancer cells: limited antiproliferative effects and absence of apoptosis associated with persistent activity of extracellular signal-regulated kinase or Akt kinase pathways. *Clin. Cancer Res.* 9, 2316-2326 (2003).

44. Sordella R, Bell DW, Haber DA *et al.*: Gefitinib-sensitizing EGFR mutations in lung cancer activate anti-apoptotic pathways. *Science* 305, 1163-1167 (2004).

• Showed that mutant EGFRs selectively activated Akt and STAT signaling pathways and the inhibition of those signals by gefitinib might contribute to the efficacy of the drug.

45. Bailey RL, Kris MG, Wolf M *et al.*: Tumor EGFR membrane staining is not clinically relevant for predicting response in patients non-small-cell lung cancer: IDEAL 1 and 2. *Proc. Am. Assoc. Cancer Res.* 44, 1362 (2003).

46. Soulieres D, Senzer NN, Vokes EE *et al.*: Multicenter Phase II study of erlotinib, an oral epidermal growth factor receptor tyrosine kinase inhibitor, in patients with recurrent or metastatic squamous cell cancer of the head and neck. *J. Clin. Oncol.* 22(1), 77-85 (2004).

47. Suzuki T, Nakagawa T, Endo H *et al.*: The sensitivity of lung cancer cell lines to the EGFR-selective tyrosine kinase inhibitor ZD1839 ('Iressa') is not related to the expression of EGFR or HER-2 or to K-*ras* gene status. *Lung Cancer* 42(1), 35-41 (2003).

• Showed that the sensitivity of lung cancer cell lines to gefitinib was not related to the expression of EGFR or HER2 or to K-*ras* gene status.

48. Hirsch FR, Franklin WA, Witta SE *et al.*: Defining gefitinib sensitivity/resistance in non-small cell lung cancer (NSCLC) cell lines by affymetrix gene arrays. *Proc. Am. Soc. Clin. Oncol.* 23, 620 (2004) (Abstract 7027).

49. Carbone DP: Tumor proteomics in targeted therapy. *40th Annual Meeting of ASCO*, New Orleans, USA (2004).

Int. J. Cancer: 112, 26–32 (2004)

Publication of the International Union Against Cancer

FAST TRACK

REDUCED EXPRESSION OF CLASS II HISTONE DEACETYLASE GENES IS ASSOCIATED WITH POOR PROGNOSIS IN LUNG CANCER PATIENTS

Hirotaka OSADA[1]*, Yoshio TATEMATSU[1], Hiroko SAITO[1], Yasushi YATABE[2], Tetsuya MITSUDOMI[3] and Takashi TAKAHASHI[1]

[1]*Division of Molecular Oncology, Aichi Cancer Center Research Institute, Nagoya, Japan*
[2]*Department of Pathology and Molecular Diagnostics, Aichi Cancer Center Hospital, Nagoya, Japan*
[3]*Division of Thoracic Surgery, Aichi Cancer Center Hospital, Nagoya, Japan*

HDAC genes are thought to be involved in gene expression through the regulation of chromatin structure, alterations of which may cause abnormal gene silencing in cancers. To clarify the possible role of HDAC genes during tumor development and progression, we studied their expression and influence on clinical features. Expression levels of HDAC class I and class II genes in cancer tissues resected from 72 patients with NSCLC were measured with real-time RT-PCR. Their association with clinicopathologic features was statistically investigated. Reduced expression of each class II HDAC gene was significantly associated with poor prognosis and an independent predictor of poor prognosis. Of all the genes, *HDAC10* was the strongest predictor of poor prognosis. Hierarchical clustering analysis showed that lung cancer tissues could be divided into 3 groups based on the expression level of class I and class II HDAC genes. The group with reduced expression of class II HDACs showed poor prognosis. These results suggest that class II HDACs may repress critical genes and that low expression of these genes may play a role in lung cancer progression. Results of clustering analyses imply that class II HDAC genes may be regulated by a similar mechanism and deregulated during cancer development.

Key words: *lung cancer; histone deacetylase; prognosis*

The accumulation of genetic and epigenetic alterations results in the development of cancer.[1,2] DNA methylation and histone modification are involved in the epigenetic alterations *via* regulating chromatin structure.[3] Histone deacetylation catalyzed by HDACs is the main histone modification in silenced gene promoters.[4] In cancer cells, several genes are silenced by alteration of the chromatin structure with or without DNA methylation,[5–7] suggesting the involvement of abnormal HDAC gene activity.[8,9] Mammalian HDACs consist of 2 groups, classical HDAC genes and the recently identified NAD-dependent SIRT family.[10,11] Based on structural and functional similarities, the classical HDAC family is divided into 2 different phylogenetic classes: class I and class II. Class I HDACs (HDACs 1–3 and 8) are most closely related to yeast RPD3, while class II HDACs (HDACs 4–7, 9 and 10) share domains with similarity to yeast HDA1. The *HDAC11* gene has been identified but not yet assigned to either class. Currently, class I HDACs are thought to be expressed in most cell types, whereas the expression pattern of class II HDACs is more restricted. Class I HDACs mainly exist in the nucleus, while class II HDACs shuttle between the nucleus and cytoplasm, responding to several signals. Nuclear export of class II HDACs is induced by phosphorylation and interaction with an adaptor protein, 14-3-3.[10,11] These fine regulations suggest that class II HDACs might be involved in cellular differentiation and developmental processes and that their dysregulation may be involved in carcinogenesis. However, alteration of the expression level and the involvement in the clinical course have not been clarified. Our study demonstrates that reduced expression of class II HDAC genes is associated with poor prognosis of lung cancer patients.

MATERIAL AND METHODS

Study population and tissue samples

Seventy-two NSCLC tissue specimens (48 adenocarcinomas, 12 squamous carcinomas, 4 adenosquamous carcinomas and 8 large cell carcinomas) were obtained with the approval of our institutional review board from 26 female and 46 male patients who had consecutively undergone potentially curative resection between January 1996 and January 1998 at Aichi Cancer Center Hospital. Twenty-two patients (31%) died (median 23 months), while 50 patients survived and were followed up for 45–75 months (median 63 months). The median age of this cohort was 62 (range 32–80) years. There were 38 pstage I, 14 pstage II and 20 pstage III/IV tumors.

Quantitative RT-PCR

RNA was isolated from tissue specimens, and random-primed cDNA samples were made using 5 μg total RNA, as described previously.[12] Expression of each HDAC gene was measured with the ABI Prism 7900 (Applied Biosystems, Foster City, CA) by quantitative RT-PCR using SYBR Green PCR Master Mix (Applied Biosystems) and cDNAs (corresponding to 20 ng of total RNA). Standard curves were made using serially diluted cDNA of one lung cancer cell line. PCR primers were designed within regions that were less similar among HDAC genes as follows: *HDAC1* F, 5′-CCTGGATACGGAGATCCCTA, and R, 5′-CCGCAAGAACTCTTCCAACT; *HDAC2* F, 5′-CCTTGATTGTGAGATTCCCA, and R, 5′-AGGAGGTCGAAGAAATGTGG; *HDAC3* F, 5′-CTCTCTGGGCTGTGATCGAT, and R, 5′-CAATCTTTGAAAACCTGAAGATG; *HDAC4* F, 5′-GAGAGACTCACCCTTCCCG, and R, 5′-CTTGGTTGGTGCAGACCGG; *HDAC5* F, 5′-AGAATGGCTTTACTGGCTCAG, and R, 5′-CATGAGCACATCCTCTATTCC; *HDAC6* F, 5′- CCTCAATCACTGAGACCATC, and R, 5′-GACTAACTCAGAGACAGCTG; *HDAC7* F, 5′-ACCCCTCAGGCTCTCATGC, and R, 5′-

Abbreviations: BRG1, BRM -related gene 1; BRM, brahma human homologue; CI, confidence interval; HDA1, histone deacetylase-A; HDAC, histone deacetylase; HP1, heterochromatin protein-1; HR, hazard ratio; MEF2, myocyte enhancer factor-2; NSCLC, non-small cell lung cancer; pstage, pathologic stage; RPD3, reduced potassium dependence 3; SIRT, homologue of silent information regulator 2.

Grant sponsor: Ministry of Education, Culture, Sports, Science and Technology of Japan; Grant sponsor: Japan Society for the Promotion of Science; Grant sponsor: Ministry of Health, Labour, and Welfare, Japan.

*Correspondence to: Division of Molecular Oncology, Aichi Cancer Center Research Institute, 1-1 Kanokoden, Chikusa-ku, Nagoya, 464-8681, Japan. Fax: +81-52-764-2993. E-mail: hosada@aichi-cc.jp

Received 12 January 2004; Accepted after revision 29 April 2004

DOI 10.1002/ijc.20395
Published online 7 June 2004 in Wiley InterScience (www.interscience.wiley.com).

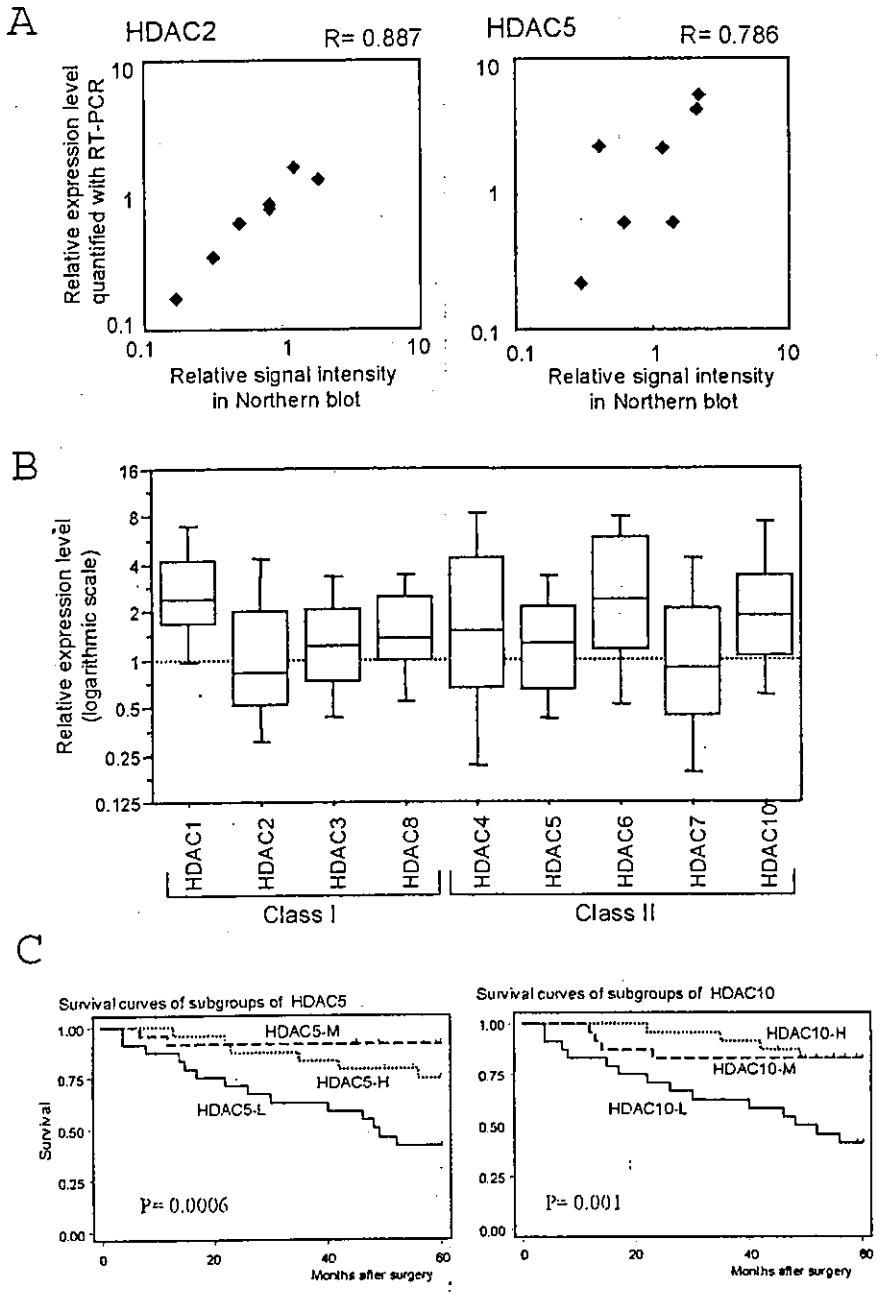


FIGURE 1 – Measurement of the expression of HDAC genes. *(a)* Consistency between Northern blot and real-time RT-PCR analyses. The intensity of Northern blot signals and relative expression level quantified by real-time RT-PCR for *HDACs 2* and *5* are compared with scatter plots. Pearson's correlation for each HDAC gene was also indicated. *(b)* Box-and-whisker plots of the expression of each HDAC gene. Boxes represent the range 25–75%, while bars in the boxes indicate the median level. Areas between lower and upper bars correspond to the 10–90% range. *(c)* Survival curves of equally trisected subgroups, high (H), moderate (M) and low (L) expression, of class II HDAC genes *HDAC5* and *HDAC10* were demonstrated. Survival curves indicate poorer prognosis of the L groups than that of the M or H groups. *p* values of log-rank analyses are also indicated.

CTGGAGCACAGGGAGCTGG; *HDAC8* F, 5′-AGGTGACGTGTCTGATGTTG, and R, 5′-CTCTGAGATCCCAGATCATG; *HDAC10* F, 5′-ATGTGGCTGTTCGGAGAGGC, and R, 5′-CTGCACTCCTGGCTGCAATG. RT-PCR analyses were performed at least twice for each HDAC gene. The expression level of each sample was normalized by the quantity of 18S rRNA and presented as a ratio to a mixed sample of normal lung tissue, the expression level of which was set at 1. Patients were divided into 3 or 2 subgroups based on the expression level of each HDAC gene.

Statistical analysis and hierarchical clustering

All statistical analyses were conducted with STATA software (StataCorp, College Station, TX). All statistical tests were 2-sided. For comparisons among subgroups, we used Pearson's χ^2 test. Log-rank tests were used to compare survival curves among the various subgroups of patients. Survival probabilities were estimated using the Kaplan-Meier method. HRs for death with 95% CIs were calculated using Cox's proportional hazards regression analysis. Associations between individual clinicopathologic variables, such as tumor histology, age, sex, smoking habit, pstage, primary tumor status (pT), nodal involvement (pN), pathologic grade of differentiation, 5-year survival and expression level of each HDAC gene, were assessed using Cox's proportional hazards regression model. A stepwise variable selection procedure was used in multivariate Cox analysis; a statistical significance level of

TABLE I – χ^2 ANALYSIS OF CLINICOPATHOLOGICAL FEATURES

Variables	HDAC5			
	H-M[1]	L	Total	*p*-value[2]
Smoking History				
Non-smoker	25	5	30	0.019*
Smoker	24	18	42	
Variables	**HDAC7**			
	H-M[1]	L	Total	*p*-value
Disease stage				
I	25	13	38	0.019*
II–III	13	21	34	
Nodal involvement				
pN = 0	29	18	47	0.038*
pN ≥ 1	9	16	25	

[1]High or moderate expression group (H-M) (≥0.8) vs. low expression group (L) (<0.8).–[2]Asterisks indicate significant *p*-values.

0.20 was used as a cut-off to determine whether a variable could be entered into or removed from the regression model. Complete linkage hierarchical clustering was performed using the Cluster program following log transformation and median centering, and the results were visualized using the TreeView program (Cluster and TreeView programs were kindly provided by Dr. M. Eisen, http://rana.lbl.gov).

Northern blot analysis

RNA samples from representative lung cancer tissues were studied with Northern blot analysis, as described previously.[12] To obtain the probes for *HDACs 1, 2, 5* and *10*, cDNA fragments of these genes were amplified by RT-PCR using the primers used for quantification, cloned into pBSSKII plasmid (Stratagene, La Jolla, CA) and sequence-verified. Signal intensity was measured with a phosphoimager BAS-2500 (Fuji, Tokyo, Japan).

RESULTS

At first, to verify the quantification with real-time RT-PCR, the signal intensity of Northern blots and the relative expression level quantified with real-time RT-PCR for *HDACs 1, 2, 5* and *10* were compared using 7 lung cancer cell line samples. Reasonable consistency (Pearson's correlation $R = 0.676–0.887$) was observed in all HDAC genes (Fig. 1*a* for *HDACs 2* and *5*). We then studied the expression of HDAC genes in 72 lung cancer specimens. The expression level of each HDAC gene varied among cancer samples (Fig. 1*b*). In analyses of the *HDAC2, -3, -8, -4, -5, -7* and *-10* genes, 25–56% of cases showed less abundant expression than normal lung. Because the *HDAC1* and *-6* genes frequently showed abundant expression, 11% and 21% of cases had lower expression of these genes than normal lung, respectively. To statistically study the possible effect of HDAC expression level, the cohort was first equally divided into 3 groups based on the expression level of each HDAC gene: high (H), moderate (M) and low (L). We investigated whether the expression level of HDAC genes may affect prognosis. Log-rank analysis indicated statistically significant differences among the survival of trisected groups in all class II HDAC genes examined (*HDACs 4–7* and *10* $p = 0.016$, 0.0006, 0.034, 0.015 and 0.001, respectively) but not class I HDAC genes (*HDACs 1–3* and *8* $p = 0.121$, 0.990, 0.150 and 0.058, respectively). Kaplan-Meier survival curves of trisected groups in all class II HDAC genes indicated significantly poorer prognosis for the L group than either the H or M group in all class II HDAC genes (Fig. 1*c* for *HDACs 5* and *10*).

Following this, we investigated whether reduced expression of HDAC genes may affect prognosis. For each HDAC gene analysis, patients with expression <80% of the level in normal lung tissues were assigned to the L group, while the remaining patients were assigned to the H-M group. The association of these 2 groups (L and H-M) and clinicopathologic features was studied using χ^2 analysis. We found a significant association of *HDAC7* with pathologic stage ($p = 0.019$) and nodal involvement ($p = 0.038$), in addition to the association between *HDAC5* and smoking history ($p = 0.019$) (Table I). Other HDAC genes did not show any significant association with clinicopathologic features, including disease stage and primary tumor status. However, we observed a significant difference in postoperative survival between the L and H-M groups of each class II HDAC gene (Fig. 2). Log-rank analysis indicated statistically significant differences in survival for these 2 groups in all class II HDAC genes examined (*HDACs 4–7* and *10* $p = 0.008$, 0.0007, 0.011, 0.016 and 0.0009, respectively) but not in class I HDAC genes (*HDACs 1–3* and *8* $p = 0.376$, 0.297, 0.710 and 0.194, respectively). Among class II HDAC genes, *HDAC5* and *HDAC10* showed the most significant difference in survival. Figure 2 shows Kaplan-Meier survival curves indicating significantly poorer prognosis for the L group than the H-M group in all class II HDAC genes.

The association of lower expression of each HDAC gene with survival was further studied with Cox's proportional hazards regression analysis (Table II). Univariate Cox analysis showed that, in all class II HDAC genes, classification into the L group was associated with poor prognosis, while class I HDAC genes did not show such an association (Table II). Among class II genes, *HDAC5* and *HDAC10* indicated the strongest association with poor prognosis (HR= 3.92 and 3.94, respectively; $p = 0.002$ for both). To study the interaction among the reduced expression of each class II HDAC gene and clinicopathologic features, multivariate Cox analysis was conducted including all clinicopathologic variables and each class II gene (Table II). These multivariate analyses showed that in all class II HDAC genes classification into the L group was associated with poor prognosis independently of any clinicopathologic feature, including pathologic stage, while class I HDAC genes did not show such an association. Among class II HDACs, the association of *HDAC5* or *HDAC10* with poor prognosis was very significant (HR= 3.91 and 4.12, $p = 0.004$ and 0.003, respectively), similar to the association between pathologic stage and poor prognosis. To study the interaction among the reduced expression of all class II HDAC genes and clinicopathological features, stepwise multivariate Cox analysis was conducted including all HDAC genes and clinicopathologic variables (Table II). In addition to pathologic stage (HR= 6.94, $p < 0.001$), reduced expression of *HDAC10* was an independent predictor of poor prognosis (HR= 7.13, $p = 0.004$), suggesting that reduced expression of *HDAC10* affects the prognosis of lung cancers most significantly and independently. The *HDAC5* gene also showed a trend, though not a significant one, toward an association with poor prognosis (HR = 2.63, $p = 0.054$).

Statistical analysis demonstrated an association between low expression of each class II HDAC gene and poor prognosis but did not provide any information on the whole picture with regard to expression of all HDAC genes in each case. The whole expression profile of all HDAC genes might indicate the number of affected HDAC genes and possibly provide information about the regulation of HDAC gene expression. To obtain the whole expression profile of all HDAC genes, we performed unsupervised hierarchical clustering analysis (Fig. 3*a*). HDAC genes occurred in 2 discrete clusters, clearly corresponding to classes I and II. Lung cancer cases also showed discrete clusters. The top cluster (cluster II, class II predominant) contained lung cancers abundantly expressing class II HDAC genes but few class I genes, while the middle cluster (cluster I, class I predominant) demonstrated the reverse pattern. The bottom cluster (cluster III) did not show any predominance; normal lung tissue belonged to this cluster. Survival among these clusters was significantly different. Kaplan-Meier survival curves in Figure 3*b* demonstrated that cluster I cases had significantly poor prognosis, while cluster II and III cases showed similarly favorable prognosis (log-rank analysis $p = 0.019$). The influence of classification into cluster I was studied with Cox's proportional hazards analysis. Univariate analysis in-

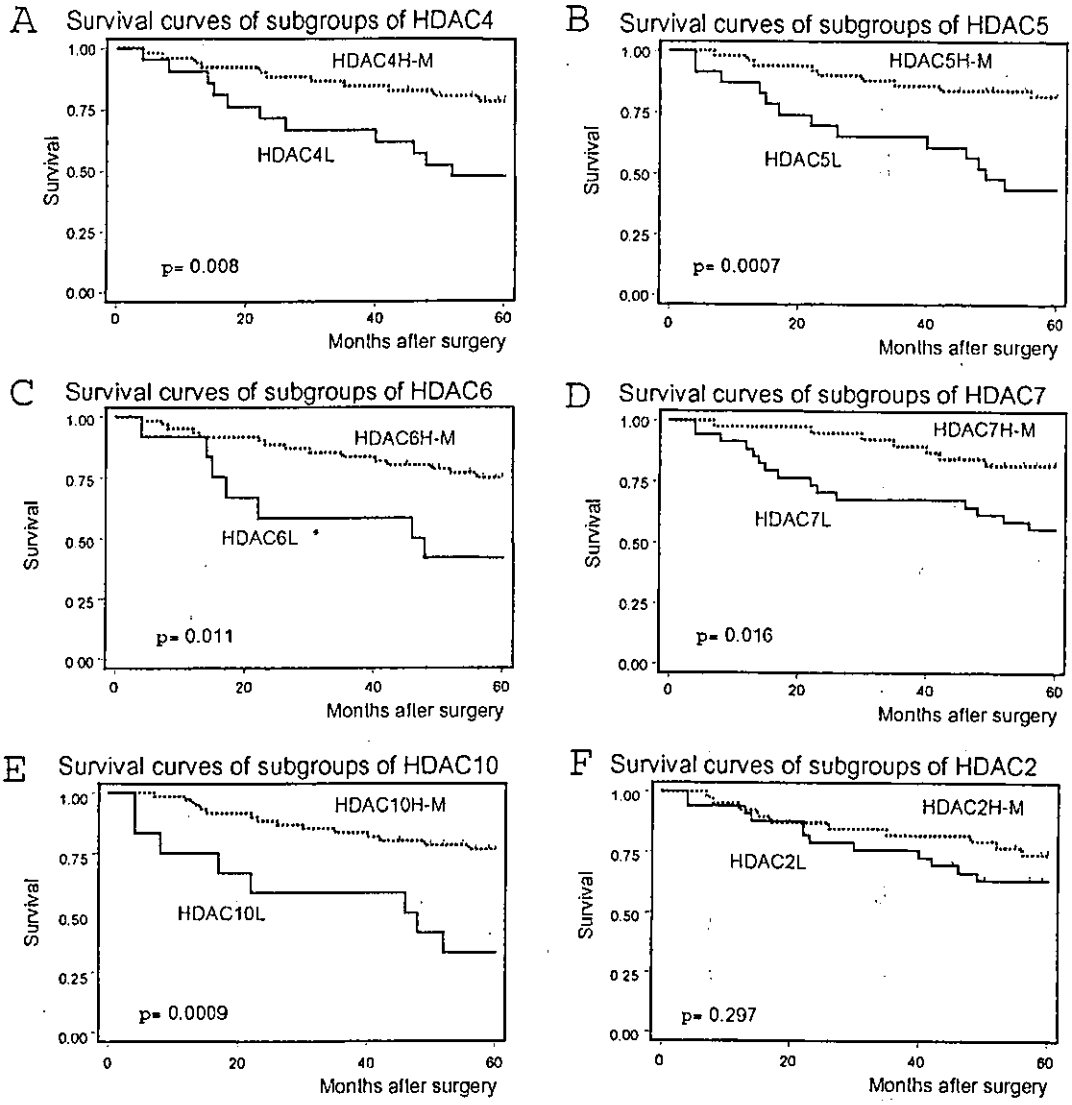


FIGURE 2 – Kaplan-Meier survival curves of subgroups of each HDAC gene. Survival curves of high or moderate expression (H-M) and low expression (L) groups of class II HDAC genes *HDAC4 (a), HDAC5 (b), HDAC6 (c), HDAC7 (d)* and *HDAC10 (e),* as well as a class I HDAC gene, *HDAC2 (f),* were demonstrated. For each HDAC gene analysis, patients with expression <80% of the level in normal lung tissue were grouped into the L group, while other patients were assigned to the H-M group. *(a–e)* Survival curves of all class II HDAC genes indicate poorer prognosis of the L groups than of the H groups. *(f)* Survival curves of the *HDAC2* groups did not show any difference in prognosis. *p* values of log-rank analyses are also indicated.

dicated significantly poor prognosis of cluster I cases (HR = 3.18, $p = 0.008$), while stepwise multivariate analysis still showed a trend, though not a significant one, toward an association between cluster I and poor prognosis ($p = 0.084$) (Table III).

DISCUSSION

Our study demonstrates that reduced expression of class II HDAC genes is associated with poor prognosis in lung cancer patients and that low expression of *HDAC10* independently affects the prognosis of lung cancer. Clustering analysis indicated a significant association between cluster I and poor prognosis. Clustering also demonstrated clear clusters of class I and class II HDAC genes, suggesting a common mechanism in transcriptional regulation in each HDAC class. The clustering of lung cancer cases demonstrated that, in most cases, expression levels of all class II HDAC genes were altered in the opposite way compared to class I genes. Class-specific expressional regulation of class II HDAC genes might be affected during lung cancer development.

Our study demonstrated statistical significance in several analyses. Generally speaking, multiple comparisons increase the probability of obtaining a statistically significant finding ($p < 0.05$) by chance alone. However, it is unlikely that our statistically significant results were obtained by chance because significant differences were observed in more than half the genes examined (5 class II of 9 HDAC genes). In addition, even if $p < 0.0055$ (= 0.05/9,

TABLE II – UNIVARIATE AND MULTIVARIATE HAZARD RATIO ACCORDING TO EXPRESSION OF HDAC GENES BY COX PROPORTIONAL HAZARDS MODEL ANALYSIS

Univariate analysis Variables	HR[1] [95% CI[2]]	Unfavorable/Favorable	*p*-value[3]
Histology	1.09 [0.37–3.21]	Squamous/Non-squamous	0.882
Age (yr)	1.80 [0.74–4.42]	≥62/<62	0.198
Gender	3.02 [1.02–8.93]	Male/Female	0.046*
Smoking	2.23 [0.87–5.70]	Smoker/Non-smoker	0.094
Disease stage	5.17 [1.90–14.05]	II–III/I	0.001*
HDAC1	1.72 [0.51–5.82]	<0.8/≥0.8[4]	0.383
HDAC2	1.56 [0.67–3.61]	<0.8/≥0.8	0.302
HDAC3	1.19 [0.48–2.91]	<0.8/≥0.8	0.710
HDAC8	1.84 [0.72–4.72]	<0.8/≥0.8	0.202
HDAC4	2.94 [1.27–6.79]	<0.8/≥0.8	0.012*
HDAC5	3.92 [1.67–9.19]	<0.8/≥0.8	0.002*
HDAC6	3.02 [1.23–7.44]	<0.8/≥0.8	0.016*
HDAC7	2.88 [1.17–7.06]	<0.8/≥0.8	0.021*
HDAC10	3.94 [1.65–9.42]	<0.8/≥0.8	0.002*

Multivariate analysis including each class II HDAC gene

Variables	HDAC genes									
	HDAC4		HDAC5		HDAC6		HDAC7		HDAC10	
	HR	*p*-value	HR	*p*-value	HR	*p*-value	HR	*p*-value	HR	*p*-value
Histology	0.65	0.470	0.92	0.897	0.74	0.608	0.72	0.574	0.66	0.511
Age (yr)	1.49	0.395	1.33	0.570	1.31	0.576	1.26	0.623	1.40	0.497
Gender	3.36	0.159	3.50	0.145	2.84	0.201	3.57	0.151	3.19	0.155
Smoking	0.83	0.805	0.60	0.516	0.94	0.932	0.79	0.760	0.86	0.830
Disease stage	3.99	0.007*	4.45	0.004*	4.20	0.005*	3.78	0.011*	4.15	0.006*
HDACs[4]	2.88	0.024*	3.91	0.004*	2.88	0.028*	2.67	0.050*	4.12	0.003*

Stepwise multivariable analysis including all HDAC genes

Variables	HR [95% CI]	Unfavorable/Favorable	*p*-value
Gender	2.87 [0.92–8.93]	Male/Female	0.069
Disease stage	6.94 [2.37–20.37]	II–III/I	<0.001*
HDAC1	0.11 [0.02–0.61]	<0.8/≥0.8	0.012*
HDAC2	2.38 [0.90–6.26]	<0.8/≥0.8	0.080
HDAC5	2.63 [0.98–7.04]	<0.8/≥0.8	0.054
HDAC10	7.13 [1.90–26.67]	<0.8/≥0.8	0.004*

[1]Hazard ratio estimated by Cox proportional hazards regression model.–[2]Confidence interval of the estimated HR.–[3]Asterisks indicate significant *p* values.–[4]Low expression group (<0.8) vs. high or moderate expression group (≥0.8).

i.e., 0.05 was divided by the number of analyzed genes) is considered statistically significant, both *HDAC5* and *HDAC10* demonstrate significant differences (*p*-values were much less than 0.0055 in Table II, Figs. 1*c*, 2*b,e*). Therefore, at least the reduced expression of *HDAC5* and *HDAC10* is associated with poor clinical outcome of lung cancer patients. However, because our cohort was rather small, these findings must be validated using training and test sets in future studies with much larger cohorts.

Class II HDAC genes are abundantly expressed in heart, skeletal muscle and brain tissue, though lung tissue also expresses class II HDAC genes moderately. *HDACs 4, 5* and *7* bind and inhibit MEF2 proteins, which play a significant transcriptional regulatory role in myogenesis.[10,11] In heart, the signal-resistant mutants of class II HDACs render cardiomyocytes resistant to hypertrophic signals, while knockout mice lacking a class II HDAC develop massive cardiac hypertrophy.[13] Therefore, class II HDACs act as signal-responsive suppressors of the transcriptional program governing cardiac hypertrophy. In addition, *in vivo* expression of a signal-resistant form of *HDAC5* resulted in sudden death accompanied by loss and morphologic changes of cardiac mitochondria as well as downregulation of mitochondrial enzymes,[14] suggesting an antiproliferative effect of class II HDAC genes. In contrast, class II HDAC molecules exhibit an antiapoptotic effect through inhibition of Nur77 induction during thymocyte development.[15] These observations suggest that class II HDAC genes may play a critical role in the differentiation and development of several tissues. In addition, class II HDACs may play a role in cancer development. Class II HDACs interact with an oncogenic molecule, Bcl-6,[16] a Kruppel-like zinc-finger repressor that is transcriptionally activated by chromosomal translocation in lymphomas. Class II HDACs also interact with the corepressor protein complex SMRT/N-CoR, which may contribute to leukemogenesis through interacting with the oncogenic chimeric molecules AML-ETO and PML-RARα.[17]

Our study showed that low expression of *HDAC5* and *HDAC10* was an independent predictor of short survival and that the cluster of low expression of class II HDAC genes was associated with poor prognosis. It is conceivable that class II HDAC genes, especially *HDAC5* and *HDAC10*, may also regulate the differentiation and proliferation of lung epithelial cells and that disruption in such regulation may be involved in lung cancer development. In this context, in our preliminary experiments, overexpression of *HDAC5* demonstrated moderate but consistent growth-inhibitory effects on lung cancer cell lines with low expression of class II HDAC genes.

HDAC5 as well as some other class II HDAC molecules, such as *HDACs 4* and *7*, contain a long N-terminal region, which interacts with MEF2.[18] When the appropriate myogenic differentiation signal is delivered, class II HDAC proteins are phosphorylated and dissociated with MEF2 and exported from the nucleus by the adaptor protein 14-3-3.[19] In airway epithelial cells, class II HDACs may also interact with transcription factors, the activity of which might be enhanced by reduced expression of class II HDACs, resulting in deregulation of differentiation and cell growth of lung epithelial cells. Therefore, identification of the transcription-regulating molecules associated with class II HDACs in lung epithelial cells is urgently required.

HDAC10 has an additional leucine-rich putative catalytic domain and is most similar to *HDAC6*, which contains 2 cata-

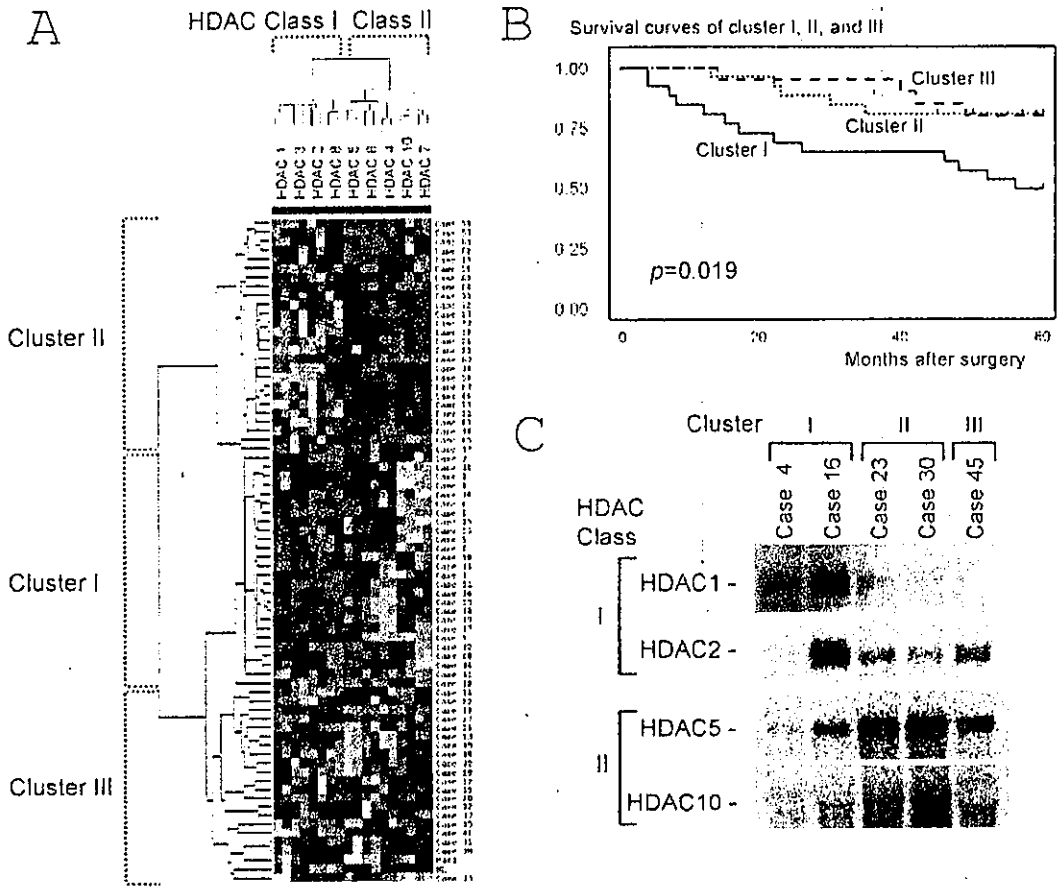


FIGURE 3 – Clustering analysis of HDAC gene expression. *(a)* Unsupervised hierarchical clustering of HDAC gene expression. The dendrogram of HDAC genes shows 2 discrete clusters, class I (*HDACs 8, 3, 2* and *1*) and class II (*HDACs 6, 4, 5, 10* and *7*). The dendrogram of lung cancer patients also indicates 3 clusters. The top cluster (cluster II, class II predominant) contained lung cancers abundantly expressing class II HDAC genes but very few class I genes, while the middle cluster (cluster I, class I predominant) demonstrated the reverse pattern. The bottom cluster (cluster III) did not show any predominance. *(b)* Survival curves of 3 clusters. Cluster I shows significantly poor prognosis, while clusters II and III show similarly favorable prognosis. *(c)* Northern blot analysis of HDAC genes in primary lung cancer specimens. Lung cancer tissues of cluster groups I and II demonstrate high expression of HDAC class I and class II genes, respectively. The lung cancer specimen of cluster III does not show such a class-specific expression pattern.

TABLE III – UNIVARIATE AND MULTIVARIATE HAZARD RATIO ACCORDING TO CLUSTER GROUPS BY COX PROPORTIONAL HAZARDS MODEL ANALYSIS

Variables	HR[1] [95% CI[2]]	Unfavorable/Favorable	*p*-value[3]
Univariate analysis			
Cluster	3.18 [1.357~7.441]	I/II–III	0.008*
Multivariate analysis			
Sex	2.51 [0.843~7.485]	Male/Female	0.098
Disease stage	3.62 [1.270~10.32]	II–III/I	0.016*
Cluster	2.18 [0.900~5.303]	I/II–III	0.084

[1]Hazard ratio estimated by Cox proportional hazards regression model.–[2]Confidence interval of the estimated HR.–[3]Asterisks indicate significant *p* values.

lytic domains. The biologic function of *HDAC10* has not been reported. However, because it interacts with class I HDACs, *HDAC2* and *HDAC3*, low expression of *HDAC10* may affect the activity of class I HDACs and enhance the imbalance of enzymatic activity between classes I and II, which might play a role in the development of cancer.

Other molecules associated with class II HDACs are reported to be involved in cancer development. Class II HDACs were reported to bind HP1α,[20] which interacts with a methyl-lysine of histone H3 and mediates gene silencing. Downregulation of HP1α expression is associated with a metastatic phenotype in breast cancers.[21] Also, subunits of chromatin-remodeling complex SWI/SNF, BRG1 and BRM, were frequently lost in lung cancers.[22] Moreover, patients with BRG1/BRM-negative lung carcinomas, independent of stage, have significantly poorer prognosis. Because HP1α interacts with BRG1[23] in addition to class II HDACs, class II HDACs, HP1α and