decrease in the electrophoretic mobility. To determine whether FTS also promoted dephosphorylation of r36 and r45, the preferred sites for phosphorylation by mTOR[31], an immunoblot was prepared with P r36/45 antibodies. Increasing FTS markedly decreased the reactivity of PHAS-I/4E-BP1 with the phosphospecific antibodies (Fig. 6A and B).

To investigate further the inhibitory effects of FTS on mTOR signaling, we determined the effect of the drug on the association of mTOR, raptor and mLST8 (Fig. 6A and B). AU1-mTOR and HA-tagged forms of raptor and mLST8 were overexpressed in 293T-cells, which were then incubated with increasing concentrations of FTS before AU1-mTOR was immunoprecipitated with anti-AU1 antibodies. Immunoblots were prepared with anti-HA antibodies to assess the relative amounts of HA-raptor and HA-mLST8 that co-immunoprecipitated with AU1-mTOR. Both HA-tagged proteins were readily detectable in immune complexes from cells incubated in the absence of FTS, indicating that mTOR, raptor and mLST8 form a complex in 293T cells. FTS did not change the amount AU1-mTOR that immunoprecipitated; however, increasing concentrations of FTS produced a progressive decrease in the amount of HA-raptor that co-immunoprecipitated. The half maximal effect on raptor dissociation from mTOR was observed at approximately 30 μM FTS (Fig. 6A, B). Results obtained with over-expressed proteins are not necessarily representative of responses of endogenous proteins. Therefore, experiments were conducted to investigate the effect FTS on the endogenous TORC1 in nontransfected cells. Similar results were found indicating the FTS blocks the association of raptor from mTOR.[2]

Incubating cells with FTS produced a stable decrease in mTOR activity that persisted even when mTOR was immunoprecipitated. The dose response curves for FTS-mediated inhibition of AU1-mTOR activity (Fig. 6C, D) and dissociation of AU1-mTOR and HA-raptor were very similar, with half maximal effects occurring between 20–30 μM. These results indicate that FTS inhibits mTOR in cells by promoting dissociation of raptor from mTORC1.

These studies provide direct evidence that FTS inhibits mTOR activity. The finding that the inhibition of mTOR activity by increasing concentrations of FTS correlated closely with the dissociation of the mTOR-raptor complex, both in cells and in vitro (Fig. 6), supports the conclusion that FTS acts by promoting dissociation of raptor from mTORC1.

Since FTS blocks both MAP kinase and mTOR, it was reasonable to conclude that it could block cell proliferation. For that reason, we conducted extensive studies to demonstrate that FTS blocks the growth of LTED cells. As shown in Figure 7A, B, FTS blocks the growth on LTED cells both in vitro and in vivo.

Our studies to date have predominantly concentrated on long term estradiol deprivation as a mode of development of resistance to aromatase inhbitors. More recently, we have examined the effect of long term tamoxifen treatment (LTTT) on MCF-7 cells. Interestingly, this maneuver also causes enhanced sensitivity to estradiol, both in vitro and in vivo.[34,35] While the up-regulation of MAP kinase is only transitory for a period of 2–3 months, these cells become hypersensitive to EGF-R mediated pathways. At the same time, we have demonstrated increased complex formation between ER alpha and the EGF-R and between ER alpha and cSRC. These studies also demonstrate that the tamoxifen resistant cells become hypersensitive to the inhibitory properties of the EGF-R tyrosine kinase inhibitor, AG 1478.

Significance of Our Findings to Development of Further Therapies

Our data suggest that cells adapt to hormonal therapy by up-regulation of growth factor pathways and ultimately become resistant to that therapy. Blockade of the pathways involved might then allow enhancement of the duration of responsiveness to various hormonal agents. Studies by Osborne and Schiff etal [36,37] and by Nicholson and his group[38,39] have

demonstrated this phenomenon both in vitro and in vivo. For example, Schiff and Osborne have treated HER-2/neu transfected MCF-7 cells with a cocktail of three kinase inhibitors: pertuzamab, gefitamab and traztuzamab as well as tamoxifen.[40] Each sequential growth factor inhibitor caused a further delay in development of resistance. Only 2/20 tumors began to regrow as a reflection of resistance when the four agents were used in combination (i.e., tamoxifen, pertuzamab, gefitimab and traztuzamab).

There are multiple agents currently in development to block growth factor pathways. Agents are available to block HER-1, 2, 3 and 4; EGF-R, IGF-R, mTOR, MAP kinase, Raf and MEK. Each of these agents might potentially be used in combination with an endocrine therapy. At the present time, this strategy is being used in several studies. A recent presentation demonstrated proof of the principle of this concept. Women with metastatic breast cancer selected to be ERα and HER-2 positive were treated either with an aromatase inhibitor alone or in combination with Herceptin. The percent of patients achieving clinical benefit (i.e., complete objective tumor regression, partial regression or stable disease for > 6 months was 27.9% percent in the aromatase inhibitor alone group and 42.9% in the combined group, a statistically significant ($p = 0.026$) finding.[41] Further studies will be necessary to determine the optimal combinations of growth factor and aromatase inhibitors in the future. However, based upon the Tandem study (examining the efficacy of aromatase inhibitor plus herceptin), this approach appears to be promising.

Synthesis of Our Current inking

Our current working model to explain adaptive hypersensitivity can be summarized as follows. Long term estradiol deprivation causes a four to ten fold up-regulation of the amount of ERα present in cell extracts and an increase in basal level of transcription of several estradiol stimulated genes. The up-regulation of the ER results from demethylation of promoter C of the ER. The lack of shift to the left in the dose response curves of these transcriptional endpoints suggested that hypersensitivity is not mediated primarily at the transcriptional level (Fig. 1 and 2). On the other hand, rapid, nongenomic effects of estradiol such as the phosphorylation of SHC and binding of SHC to ERα are easily demonstrable and appear enhanced in the LTED cells. Taken together, these observations suggest that adaptive hypersensitivity is associated with an increased utilization of nongenomic, plasma membrane mediated pathways. is results in an increased level of activation of the MAP kinase as well as the PI-3-kinase and mTOR pathways. All of these signals converge on downstream effectors which are directly involved in cell cycle functionality and which probably exert synergistic effects at that level. As a reflection of this synergy, E2F1, an integrator of cell cycle stimulatory and inhibitory events, is hypersensitive to the effects of estradiol in LTED cells.[7] us, our working hypothesis at present is that hypersensitivity reflects upstream nongenomic ERα events as well as downstream synergistic interactions of several pathways converging at the level of the cell cycle.

It is clear that primary endocrine therapies can exert pressure on breast cancer cells that causes them to adapt as a reflection of their inherent plasticity. Based upon this concept, we postulate that certain patients may become resistant to tamoxifen as a result of developing hypersensitivity to the estrogenic properties of tamoxifen. Up-regulation of growth factor pathways involving erb-B-2, IGF-1 receptor and the EGF receptor are associated with this process.[2] The estrogen agonistic properties of tamoxifen under these circumstances might explain the superiority of clinical responses in patients receiving aromatase inhibitors as opposed to tamoxifen. It is possible to counteract the effects of the adaptive processes leading to growth factor up-regulation. If breast cancer cells are exceedingly sensitive to small amounts of estradiol or to the estrogenic properties of tamoxifen, one therefore needs highly potent aromatase inhibitors to block estrogen synthesis or pure antiestrogens such as fulvestrant.

Blockade of the downstream effects of the IGF-1-R, EGF-R and erb-B-2 pathways would also be beneficial and allow continuing responsiveness to aromatase inhibitors or tamoxifen.

Disruption of each of several key steps could reduce the level of sensitivity to estradiol and block cell growth. Figure 8 illustrates the potential sites for disruption of adaptive hypersensitivity. An agent that blocks the nodal points through which several growth factor pathways must pass might be a more suitable therapy than combination of several growth factor blocking agents. Our preliminary data suggest that FTS blocks two nodal points, the functionality of Ras and the activity of mTOR. FTS also effectively inhibits the proliferation of MCF-7 breast cancer cells in culture. Since this agent blocks MAP kinase as well as mTOR, it may be ideal for the prevention of adaptive hypersensitivity and prolongation of the effects of hormonal therapy in breast cancer. We are currently conducting further studies in xenograft models to demonstrate its efficacy. We envision the possibility that women with breast cancer will receive a combination of aromatase inhibitors plus FTS. In this way, the beneficial effects of the aromatase inhibitor may be prolonged and relapses due to growth factor over-expression might be prevented or retarded.

Acknowledgements

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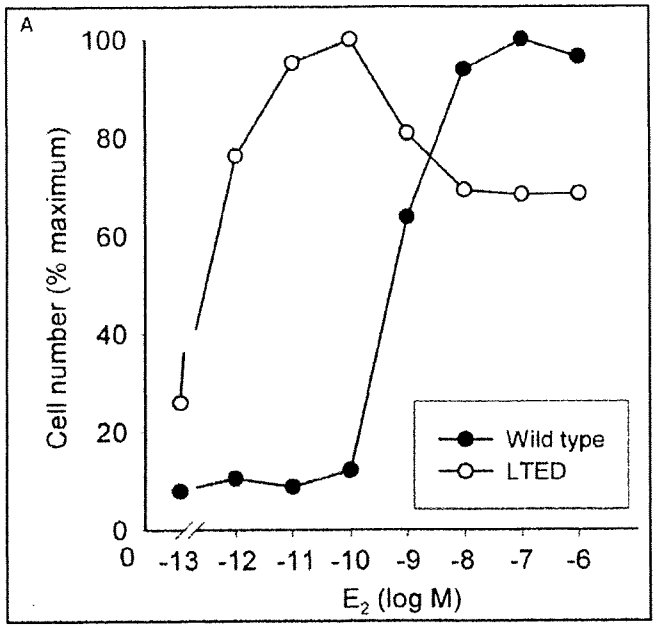
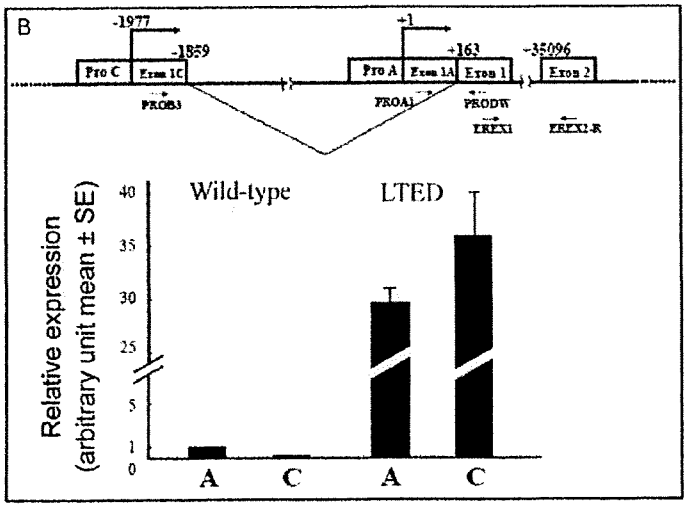
References

1. Santen RJ, Manni A, Harvey H, et al. Endocrine treatment of breast cancer in women. Endocr Rev 1990;11(2):221–265. [PubMed: 2194783]
2. McMahon LP, Yue W, Santen RJ, et al. Farnesylthiosalicylic acid inhibits mammalian target of rapamycin (mTOR) activity both in cells and in vitro by promoting dissociation of the mTOR-raptor complex. J Mol Endocrinol 2005;19(1):175–183.
3. Santen RJ, Song RX, Zhang Z, et al. Long-term estradiol deprivation in breast cancer cells up-regulates growth factor signaling and enhances estrogen sensitivity. Endocr Relat Cancer 2005;12(Suppl 1):S61–73. [PubMed: 16113100]
4. Shim WS, DiRenzo J, DeCaprio JA, et al. Segregation of steroid receptor coactivator-1 from steroid receptors in mammary epithelium. Proc Natl Acad Sci USA 1999;96(1):208–13. [PubMed: 9874797]
5. Shim WS, Conaway M, Masamura S, et al. Estradiol hypersensitivity and mitogen-activated protein kinase expression in long-term estrogen deprived human breast cancer cells in vivo. Endocrinology 2000;141(1):396–405. [PubMed: 10614662]
6. Yue W, Wang J, Li Y, et al. Farnesylthiosalicylic acid blocks mammalian target of rapamycin signaling in breast cancer cells. Int J Cancer 2005;117(5):746–754. [PubMed: 15957161]
7. Song RX. Membrane-initiated steroid signaling action of estrogen and breast cancer. Seminars in Reproductive Medicine May;2007 25(3):187–97. [PubMed: 17447208]
8. Song RX, Fan P, Yue W, Chen Y, Santen RJ. Role of receptor complexes in the extranuclear actions of estrogen receptor alpha in breast cancer. Endocrine-Related Cancer 2006 Dec;13(Suppl 1):S3–13. [PubMed: 17259556]
9. Yue W, Wang JP, Conaway M, et al. Activation of the MAP Kinase pathway Enhances Sensitivity of MCF-7 Breast Cancer Cells to the Mitogenic Effect of Estradiol. Endocrinology 2002;143(9):3221–9. [PubMed: 12193533]
10. Yue W, Wang JP, Conaway MR, et al. Adaptive hypersensitivity following long-term estrogen deprivation: involvement of multiple signal pathways. J Steroid Biochem Mol Biol 2003;86(3–5): 265–74. [PubMed: 14623520]
11. Jeng MH, Yue W, Eischeid A, et al. Role of MAP kinase in the enhanced cell proliferation of long term estrogen deprived human breast cancer cells. Breast Cancer Res Treat 2000;62(3):167–175. [PubMed: 11072781]

12. Masamura S, Santner SJ, Heitjan DF, et al. Estrogen deprivation causes estradiol hypersensitivity in human breast cancer cells. J Clin Endocrinol Metab 1995;80(10):2918–2925. [PubMed: 7559875]
13. Jeng MH, Shupnik MA, Bender TP, et al. Estrogen receptor expression and function in long-term estrogen-deprived human breast cancer cells. Endocrinology 1998;139(10):4164–74. [PubMed: 9751496]
13a. Sogon T, Masamura S, Hayashi S-I, et al. J Steroid Biochem Mol Biol. 2007
14. Pelicci G, Lanfrancone L, Salcini AE, et al. Constitutive phosphorylation of SHC proteins in human tumors. Oncogene 1995;11(5):899–907. [PubMed: 7675449]
15. Pelicci G, Dente L, De Giuseppe A, et al. A family of SHC related proteins with conserved PTB, CH1 and SH2 regions. Oncogene 1996;13(3):633–641. [PubMed: 8760305]
16. Yue W, Wang JP, Li Y, et al. Farnesylthiosalicylic acid blocks mammalian target of rapamycin signaling in breast cancer cells. Int J Cancer 2005;117(5):746–54. [PubMed: 15957161]
17. Migliaccio A, Di Domenico M, Castoria G, et al. Tyrosine kinase/p21ras/MAP-kinase pathway activation by estradiol-receptor complex in MCF-7 cells. EMBO J 1996;15(6):1292–1300. [PubMed: 8635462]
18. Kelly MJ, Lagrange AH, Wagner EJ, et al. Rapid effects of estrogen to modulate G protein-coupled receptors via activation of protein kinase A and protein kinase C pathways. Steroids 1999;64(1–2): 64–75. [PubMed: 10323674]
19. Valverde MA, Rojas P, Amigo J, et al. Acute activation of Maxi-K channels (hSlo) by estradiol binding to the beta subunit [see comments]. Science 1999;285(5435):1929–1931. [PubMed: 10489376]
20. Song RX, McPherson RA, Adam L, et al. Linkage of rapid estrogen action to MAPK activation by ERalpha-SHC association and SHC pathway activation. J Mol Endocrinol 2002;16(1):116–127.
21. Dikic I, Batzer AG, Blaikie P, et al. SHC binding to nerve growth factor receptor is mediated by the phosphotyrosine interaction domain. J Biol Chem 1995;270(25):15125–15129. [PubMed: 7541035]
22. Boney CM, Gruppuso PA, Faris RA, et al. The critical role of SHC in insulin-like growth factor-I-mediated mitogenesis and differentiation in 3T3-L1 preadipocytes. J Mol Endocrinol 2000;14(6): 805–813.
23. Collins P, Webb C. Estrogen hits the surface. [see comments] Nature Medicine 1999;5(10):1130–1131.
24. Watson CS, Campbell CH, Gametchu B. Membrane oestrogen receptors on rat pituitary tumour cells: immuno-identification and responses to oestradiol and xenoestrogens. [Review] [45 refs]. Exp Physiol 1999;84(6):1013–1022. [PubMed: 10564698]
25. Watson CS, Norfleet AM, Pappas TC, et al. Rapid actions of estrogens in GH3/B6 pituitary tumor cells via a plasma membrane version of estrogen receptor-alpha. Steroids 1999;64(1–2):5–13. [PubMed: 10323667]
26. Duan R, Xie W, Burghardt RC, et al. Estrogen receptor-mediated activation of the serum response element in MCF-7 cells through MAPK-dependent phosphorylation of Elk-1. J Biol Chem 2001;276 (15):11590–11598. [PubMed: 11145955]
27. Roberson MS, Misra-Press A, Laurance ME, et al. A role for mitogen-activated protein kinase in mediating activation of the glycoprotein hormone alpha-subunit promoter by gonadotropin-releasing hormone. Mol Cell Biol 1995;15(7):3531–3539. [PubMed: 7791760]
28. Song RX, Barnes CJ, Zhang Z, et al. The role of SHC and insulin-like growth factor 1 receptor in mediating the translocation of estrogen receptor alpha to the plasma membrane. Proc Natl Acad Sci USA 2004;101(7):2076–81. [PubMed: 14764897]
29. Song, RX.; Santen, RJ. Role of IFG-1R in mediating nongenomic effects of estrogen receptor alpha. Paper presented at: The Endocrine Society's 85th Annual Meeting (USA); Philadelphia. 2003;
29a. Levin E. J Biol Chem.
30. Haklai R, Weisz MG, Elad G, et al. Dislodgment and accelerated degradation of Ras. Biochemistry 1998;37(5):1306–14. [PubMed: 9477957]
31. Harris TE, Lawrence JC Jr. TOR signaling. [Review] [221 refs]. Science's Stke [Electronic Resource]. Sci STKE. 2003;(212)ref [15]

32. Lawrence JC Jr, Brunn GJ. Insulin signaling and the control of PHAS-I phosphorylation. [Review] [102 refs]. Prog Mol Subcell Biol 2001;26:1–31. [PubMed: 11575163]
33. Brunn GJ, Hudson CC, Sekulic A, et al. Phosphorylation of the translational repressor PHAS-I by the mammalian target of rapamycin. Science 1997;277(5322):99–101. [PubMed: 9204908]
34. Berstein L, Zheng H, Yue W, et al. New approaches to the understanding of tamoxifen action and resistance. Endocr Relat Cancer 2003;10(2):267–77. [PubMed: 12790788]
35. Fan P, Wang J, Santen RJ, et al. Long-term treatment with tamoxifen facilitates translocation of estrogen receptor alpha out of the nucleus and enhances its interaction with EGFR in MCF-7 breast cancer cells. Cancer Res 2007;67(3):1352–1360. [PubMed: 17283173]
36. Osborne CK, Hamilton B, Titus G, et al. Epidermal growth factor stimulation of human breast cancer cells in culture. Cancer Res 1980;40(7):2361–2366. [PubMed: 6966967]
37. Osborne CK, Fuqua SA. Mechanisms of Tamoxifen Resistance. Breast Cancer Res Treat 1994;32:49–55. [PubMed: 7819585]
38. Hiscox S, Morgan L, Green TP, et al. Elevated Src activity promotes cellular invasion and motility in tamoxifen resistant breast cancer cells. Breast Cancer Res Treat 2006;97(3):263–274. [PubMed: 16333527]
39. Hiscox S, Morgan L, Barrow D, et al. Tamoxifen resistance in breast cancer cells is accompanied by an enhanced motile and invasive phenotype: inhibition by gefitinib ('Iressa', ZD1839). Clin Exp Metastasis 2004;21(3):201–212. [PubMed: 15387370]
40. Schiff R, Massarweh SA, Shou J, et al. Advanced concepts in estrogen receptor biology and breast cancer endocrine resistance: implicated role of growth factor signaling and estrogen receptor coregulators. [Review] [97 refs]. Cancer Chemother Pharmacol 2005;56(Suppl 1):10–20. [PubMed: 16273359]
41. Mackey JR, Kaufman B, Clemens M, et al. Trastuzumab prolongs progression-free survival in hormone dependent and HER2-positive metastatic breast cancer. Breast Cancer Res Treat 2006;100 (Suppl 1)S5, Ab 3





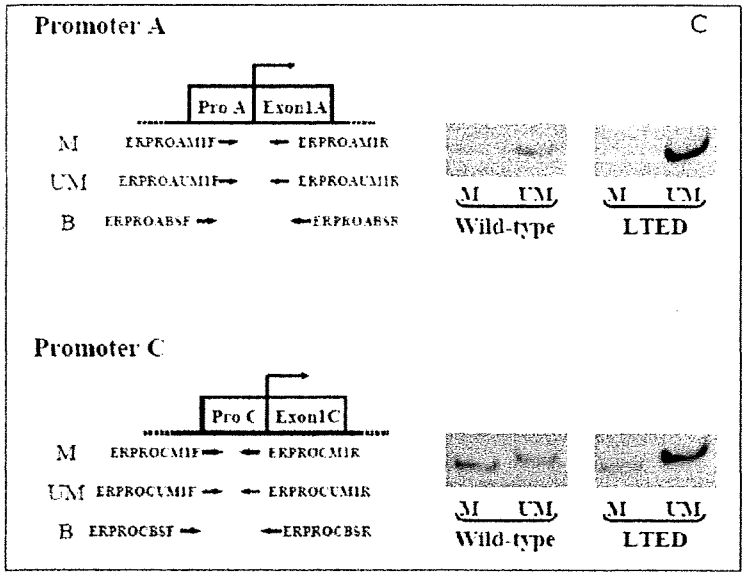


Figure 1.

A) E_2-induced cell proliferation. Wild type MCF-7 and LTED cells were plated in 6 well plates at a density of 60,000 cells/well. After 2 days the cells were refed with phenol-red and serum free IMEM (improved modified Eagles medium) and cultured in this medium for another 2 days before treatment with various concentrations of E_2 in the presence of ICI 182,780 (fulvestrant) at a 1 nmol concentration to abrogate the effects of any residual estradiol in the medium. Cell number was counted 5 days after treatment.[7,9] From: Yue W et al. Endocrinology 2002; 143(9):3221-9;[9] with permission of The Endocrine Society. B) Schematic representation of a part of ER alpha gene organization is shown. The transcription start site of Promoter A is defined as +1. Relative expression of ER alpha mRNA from promoters A and C in wild type and LTED cells is shown. Expression levels of ER alpha mRNA from promoters A and C were quantified by RT-PCR. C) COBRA assay for gene promoter C of ER α in wild type and LTED cells: an image of the polyacrilamide gel showing the methylated (M) and unmethylated (UM) products. B,C) From: Sogon T et al. J Steroid Biochem Mol Biol 2007; 105(1–5):106–14;[13a] with permission of Elsevier.

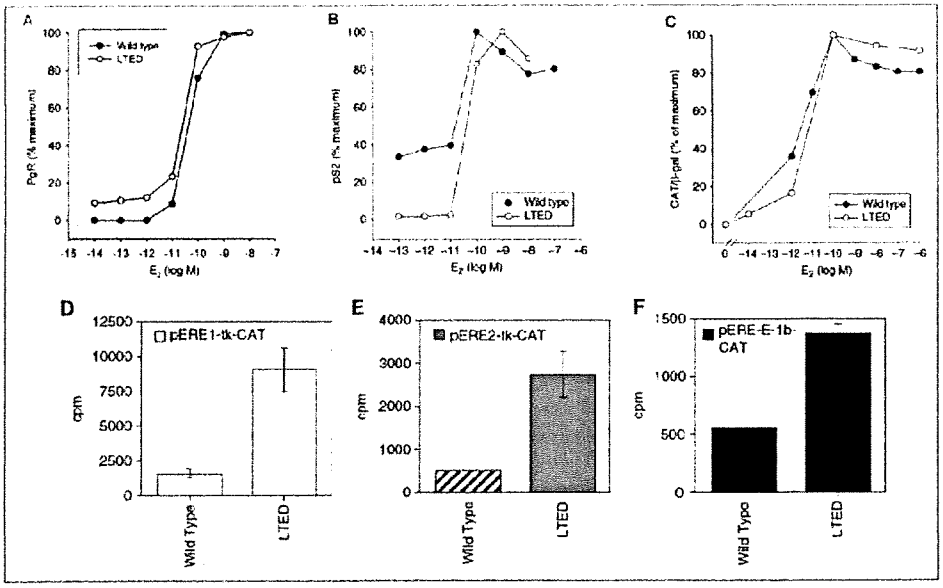


Figure 2.

A–C) Wild–type MCF-7 and LTED cells, deprived of E_2, were treated with different concentrations of E_2. Cytosols were measured for PgR (A), pS2 protein (B) and ERE-TK-CAT activity (C) 48 h after E_2 treatment. A–C) From: Yue W et al. Endocrinology 2002; 143(9): 3221-9;[9] with permission of The Endocrine Society. D–F) ER trans-activation function in wild-type MCF-7 and LTED cells under basal conditions. Wild type and LTED cells were deprived of estrogen and transfected with ERE-TK-CAT (D), pERE-2-TK-CAT E) or pERE-E1b-CAT (F) reporter plasmids in conjunction with pCMV-beta Gal plasmid as internal control. Two days later, cell cytosols were collected and assayed for CAT activities using the same amount of beta-galactosidase units.[9,11,13] D–F) From: Jeng MH et al. Endocrinology 1998; 139(10): 4164–74;[13] with permission of The Endocrine Society.

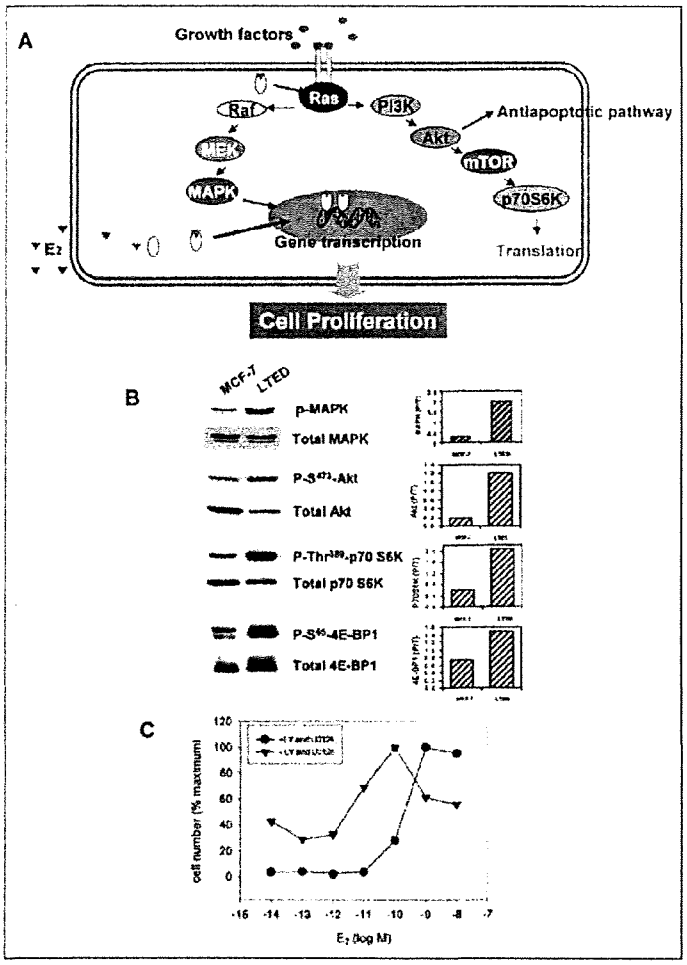


Figure 3.

A) Diagrammatic representation of the MAP kinase and PI-3-kinase signaling pathways activated when growth factors bind to their trans-membrane receptors. After auto-phosphorylation of the receptor, a series of events occurs which results in the activation of Ras. Downstream from Ras is the activation of the MAP kinase pathway with its components Raf and Mek and the activation of the PI-3-kinase pathway with its downstream components Akt, mTOR and p70S6K. At the same time, estradiol binds to the estrogen receptor and initiates transcription in the nucleus. B, top) Comparison of total and activated MAP kinase, detected with a phosphospecific antibody directed against activated MAP kinase and an antibody directed against total MAP kinase, in WT (wild-type MCF-7) and LTED cells. The right portion of the panel is a quantitation of the ratio of activated to total MAP kinase in WT and LTED cells.[16] B, second, third and fourth panels) Use of phosphospecific antibodies to quantitate the levels of activated Akt (second panel), p70S6 kinase (third panel) and 4E-BP1 (fourth panel) in wild type MCF-7 and LTEDS cells.[16] C) Treatment of LTED cells with an inhibitor of MAP kinase (U-0126) and PI-3- kinase (LY 292004) to demonstrate a shift to the right of LTED cells to a normal level of sensitivity to estradiol.[7,9] From: Yue W et al. J Steroid Biochem Mol Biol 2003; 86(3–5):265–74;[8] ©2003 with permission from Elsevier.

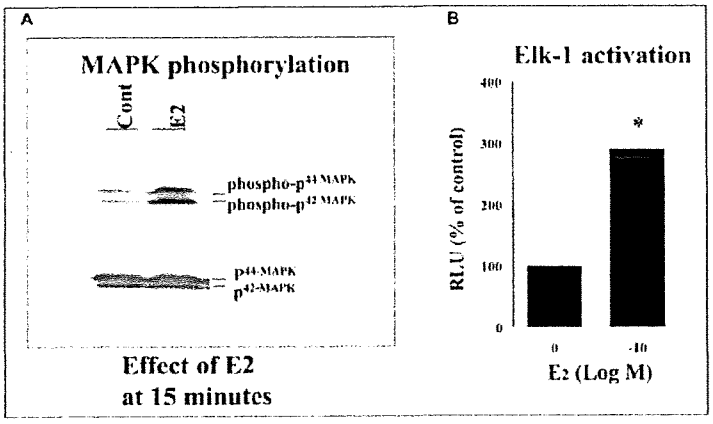


Figure 4.

A) Effect of 0.1 nM estradiol on levels of activated and total MAP kinase measured 15 min after addition of steroid. Shown on the top segment is activated MAP kinase as assessed by an antibody specific for activated MAP kinase and on the bottom segment, total MAP kinase. B) Effect of 0.1 nM estradiol on the activation of ELK-1.

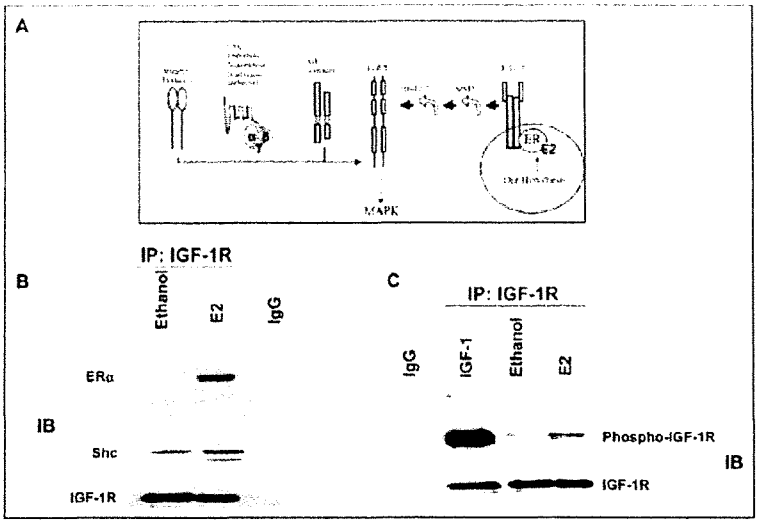


Figure 5.

A, top) Diagrammatic representation of a model in which estradiol binds to ERα which then binds to the adaptor protein, SHC. At the same time estradiol causes phosphorylation of the IGF-1-R, which provides a binding site for SHC. In this model, estradiol signals through the IGF-1-R and activates MAP kinase which then acts through Elk-1 to initiate gene transcription. B) estradiol-induced protein complex formation among ERα, SHC and IGF-1-R. MCF-7 cells were treated with vehicle, 1 ng/ml IGF-1, or E_2 at 0.1 nM for the times indicated. Lysates were immunoprecipitated with IGF-1-R antibody. The nonspecific monoclonal antibody (IgG) served as a negative control.[28] C) estradiol increases the phosphorylation of the IGF-1-R.

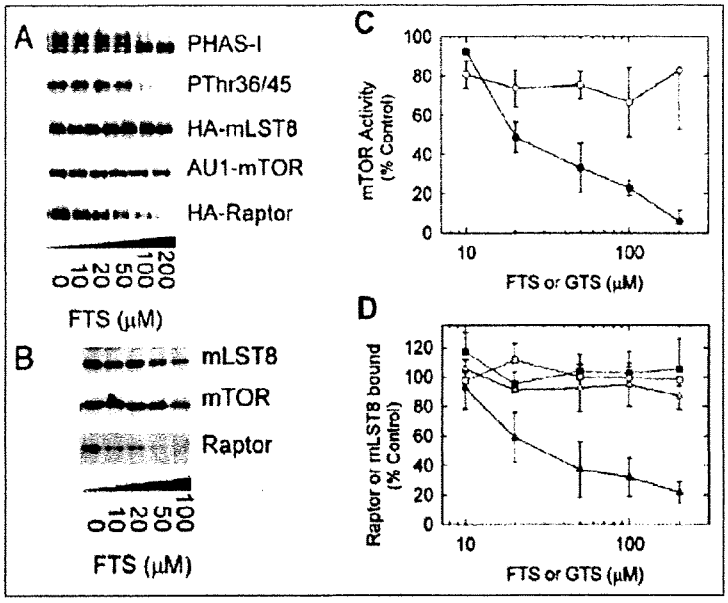


Figure 6.

Left) FTS promotes raptor dissociation and inhibits mTOR activity in cell extracts. A) 293T cells were transfected with pcDNA3 alone (vector) or with a combination of pcDNA3-AU---mTOR, pcDNA3-3-HA-raptor and pcDNA3-3HA-mLST8. Extracts of cells were incubated with increasing concentration of FTS for 30 min before AU-1-mTOR was immunoprecipitated. Samples of the immune complexes were incubated with (γ32P)-ATP and recombinant (HIS 6) PHAS-1 and then subjected to SDS-PAGE. A phosphor image of a dried gel was obtained to detect ^{32}P-PHAS1 and an immunoblot was prepared with PThr36/45 antibodies. Other samples of the immune complexes were subjected to SDS-PAGE and immunoblots were prepared with antibodies to the HA epitope or to mTOR.[2] B) Extracts of nontransfected 293T cells were incubated with increasing concentrations of FTS before mTOR was immunoprecipitated with mTab 1. A control immunoprecipitation was conducted using nonimmune IgG(Nl). Immune complexes were subjected to SDS-PAGE and immunoblots were prepared with antibodies to mLST8, mTOR and raptor.[2] Right) Relative effects of increasing concentrations of FTS and GTS on mTOR activity and the association of mTOR and raptor. Samples of extracts from 293T cells overexpressing AU1-mTOR, HA-raptor and HA-mLST8 were incubated for 1 hr with increasing concentrations of FTS (●, ◆, ■) or GTS (○, Δ, □) before immunoprecipitations were conducted with anti-AU 1 antibodies.[2] A) mTOR kinase activity (●, ○) was determined by measuring ^{32}P incorporation into (HIS6) PHAS-1 in immune complex kinase assays performed with (γ32P)-ATP. B) The relative amounts of HA-raptor (◆,○) and HA-mLST8 (■, Δ) that co-immunoprecipitated with AU-1-mTOR were determined after immunoblotting with anti-HA antibodies. The results (mean values ± SE for five experiments) are expressed as percentages of the mTOR activity (C) or co-immununoprecipitating proteins (D) from samples incubated without FTS or GTS and have been corrected for the amounts of AU-1–mTOR immunopecipitated.[2] From: McMahon LP et al. J Mol Endocrinol 2005; 19(1):175–183;[2] with permission of The Endocrine Society.

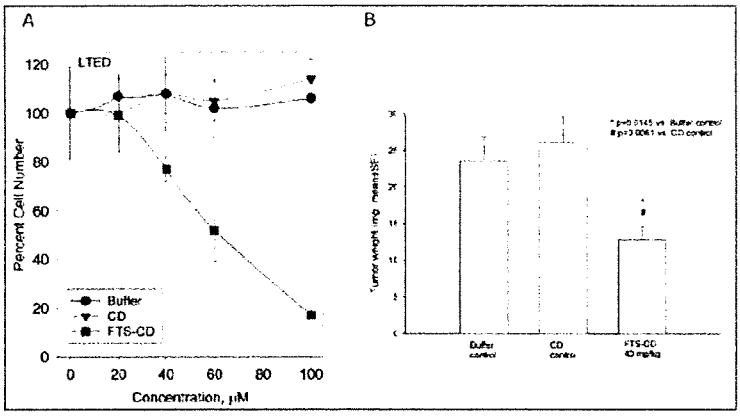


Figure 7.

A) In vitro effects of FTS on cell growth. Effects of FTS complexed with cyclodextrin (CD) for solubility were compared with buffer or cyclodextrin (CD) alone on the number of LTED cells expressed as a percent of maximum number. The ordinate shows the concentration of FTS used. B) In vivo effects of FTS on cell growth. LTED cells were implanted into castrate nude mice to form xenografts. Silastic implants delivering estradiol at amounts sufficient to provide plasma levels of estradiol of 5 pg/ml were implanted. One group received buffer alone, the second cyclodextrin alone and the third FTS 40 mg/kg complexed to cyclodextrin. The effects of FTS-CD compared to CD control were statistically significant at $p = 0.0061$.

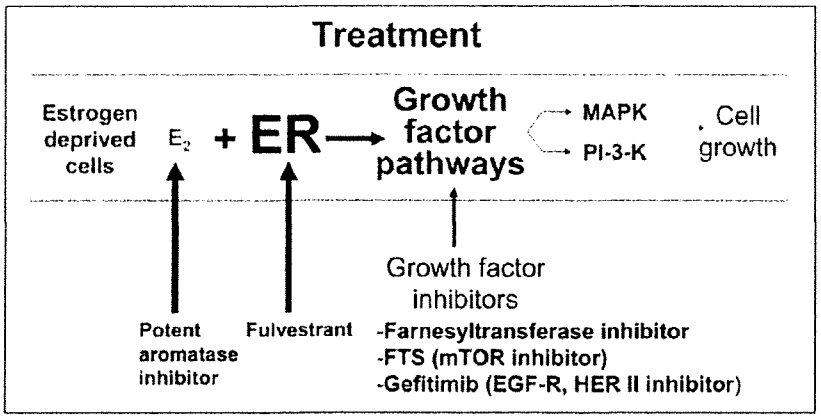


Figure 8.

Practical implications of the effects of up-regulation of growth factor pathways and development of hypersensitivity to estradiol. Potent aromatase inhibitors are useful to counteract the enhanced sensitivity to estradiol resulting from adaptation to prolonged estradiol deprivation. A pure antiestrogen such as fulvestrant can counteract the up-regulation of the ER that occurs. Growth factor inhibitors such as FTS, farnesyl-transferase inhibitors and growth factor inhibitors such as Iressa and others can be used to block up-regulation of growth factor pathways.

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Soluble Fas level and cancer mortality: Findings from a nested case–control study within a large-scale prospective study

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Soluble Fas (sFas) is known to play an important role in the development of cancers of various sites. To confirm whether or not the serum sFas level can be a predictor of cancer, we conducted a nested case–control study within a large-scale population-based cohort study in Japan. Serum samples were collected from 39,242 participants (13,839 men and 25,403 women) at baseline, all of whom were followed until 1997 for mortality and until 1994 for cancer incidence. Three controls were randomly selected and matched to each cancer case for gender, age and residential area. Serum values of sFas were measured by enzyme-linked immuno-adsorbent assay, using commercially available kits. The odds ratios (ORs) and 95% confidence intervals (95% CIs) were estimated using conditional logistic models, based on 798 total cancer mortality cases and their 2,353 matched controls. The risk of total cancer mortality was increased according to sFas levels, and the OR of the highest quartile compared with that of the lowest was 1.81 (95% CI; 1.40–2.34) after adjusting for smoking and drinking status, and body mass index. This positive association remained unaltered when cases were divided into 2 groups according to the observation period. Our results suggest that serum sFas has a possibility to detect people at high risk for cancer prior to diagnosis, since it increased before cancer diagnosis in those apparently healthy people.

Key words: soluble Fas; cancer; cohort study; nested case–control study

Fas on membrane of many cells induces cell apoptosis by binding to the Fas ligand that is expressed predominantly in activated T cells. This interaction between Fas and Fas ligand is known to play an important role in the spontaneous death of cancer cells induced by the immune system. Another form of Fas, circulating soluble Fas (sFas), antagonizes the cell-surface Fas function and may offer a survival advantage to cells. Previous studies have demonstrated that sFas levels were increased in cancer patients from many sites.[1–5] Furthermore, the overexpression of sFas in serum is correlated with a cancer stage and/or prognosis.[1,6,7] Given that much of the evidence is derived from case–control studies, it is plausible that an elevation of the sFas level in serum might contribute to a tumor escaping from Fas-induced apoptosis. However, it remains unclear whether individuals with high sFas levels are at an increased risk of cancer, *i.e.*, whether or not sFas levels can predict cancer occurrence. Only 1 paper[8] has examined the relationship between sFas levels and ovarian cancer in a nested case–control study. The authors failed to find any association between serum sFas levels and ovarian cancer occurrences, which were diagnosed at an average of 5.1 years after blood was drawn. To test the hypothesis that elevated serum sFas could be a predictive marker of cancer and to identify people at risk of cancer, we examined the relationship between serum sFas levels and the risk of cancer mortality in a nested case–control study within a large-scale population-based cohort study.

Material and methods

Study population and serum samples

The Japan Collaborative Cohort Study (JACC Study), conducted from 1998 to 1990 using a self-administered questionnaire on lifestyle factors, consisted of 110,792 subjects aged 40–79 years living in 45 municipalities, across Japan. At baseline, ~35% of the cohort participants donated blood samples. Details of the study design and methods were previously described.[9,10] In brief, serum samples were stored in deep freezers at −80°C until 1999. Prior to the measurement of serum components, we macroscopically examined the condition of all sera and found that serum samples of 39,242 participants (13,839 men and 25,403 women) were

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TABLE I – SITES OF CANCER CASES

		Mortality	
Total cancer		798	100.0
Oesophagus	C15	22	2.8
Stomach	C16	152	19.0
Colon	C18	60	7.5
Rectum	C20	31	3.9
Liver and intrahepatic bile ducts	C22	64	8.0
Gallbladder, other and unspecified parts of biliary tract	C23–24	65	8.1
Pancreas	C25	58	7.3
Bronchus and lung	C34	162	20.3
Breast	C50	11	1.4
Prostate	C61	19	2.4
Hodgkin's disease and lymphoma	C81–85	21	2.6
Myeloma	C90	12	1.5
Leukemia	C91–95	14	1.8
Other		107	13.4

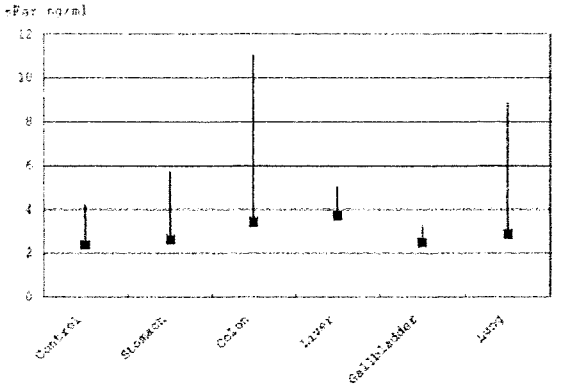

FIGURE 1 – Mean values and standard deviation of sFas among major sites of cancer and control.

suitable for biochemical analysis. Also, comparison of fresh and stored samples of pooled serum prepared for quality control demonstrated that serum levels of proteins remained steady (mean differences of total protein and albumin were around 5.0%) when frozen at −80°C for 6 years.[11] The study design and use of serum were approved by the Ethical Board at the Nagoya University School of Medicine, where the central office of the JACC Study was located at the time.

Follow-up

The causes and dates of death among the study subjects were annually or biannually determined by reviewing all death certificates in each study area with the permission of the Director-General of the Prime Minister's Office (Ministry of Public Management, Home Affairs, Post and Telecommunications) till 1997. Participants who had moved out of their study areas at baseline were also identified with administrative assistance from public health nurses in each area by reviewing the population-register sheets of cohort members. Cancer incidences, including primary cancer site, histological category, and date of incidence were also identified in 24 areas out of 45 till 1994.

Selection of cases and controls (Nested case–control study)

We found 2,142 deaths from all-causes, up to 1997, and 764 cancer incident cases up to 1994 among subjects whose sera were available at baseline. For each case, we randomly selected 3–4 controls, matching them for gender, age (as near as possible) and residential area. Thus, 2,630 cases and 10,205 controls were selected, and their serum sFas values were measured.

Biochemical assays of sera

In 1999 and 2000, serum values of sFas were measured by enzyme-linked immuno-adsorbent assay, using commercially available kits (MBL Co., Nagoya). All samples were assayed at a single laboratory (SRL, Hachioji) by trained staff who had no knowledge of case or control identities. Assay methods have been described in detail elsewhere.[11] Since sFas values were systematically low in 1 area, we excluded all sera from that area from the analysis. This exclusion left 2,393 cases (deaths from all-causes and cancer incidence) and 9,279 controls, from among whom we found 798 cancer mortality cases and their 2,353 matched controls eligible for the present study. Mean day-to-day variations (inter-assay precision) of the quality control samples at the time of measurement for our samples was 7.9%.[11]The range of the assay for serum sFas level was 1.0–10 ng/ml; the intra- and inter-assay precisions were 2.1–5.5% and 8.2–12.3%, respectively.[11]

Analytical method

Baseline characteristics were compared between cases and controls using the Mantel Haenszel test. Cutoff points of sFas levels were determined according to the distribution of all controls whose sFas value was assayed. Odds ratios (ORs) and their 95% confidence intervals (CIs) for total cancer mortality associated with serum sFas levels adjusted for age were estimated using conditional logistic models. Tests for trends were conducted by entering ordinal sFas values.

We also conducted additional analyses to determine potential confounders such as smoking (current smokers, not current smokers and unknown), alcohol consumption (habitual drinkers, non-drinkers and unknown) and body mass index (BMI; <18.5, 18.5–24.9, 25.0≤, and unknown). BMI at baseline was calculated based on the height and weight reported in the questionnaire survey (BMI = weight in kilograms/[height in meters]^2). To examine the influence of undiagnosed cancer at baseline on the association of serum sFas levels and cancer risk, we conducted analyses stratified by the observation period from baseline to 1994 and beyond. All p values were 2-sided, and all statistical analyses were performed using the Statistical Analysis System (SAS 9.1, Cary, NC).

Results

The number of cancer mortalities at specific sites is shown in Table I. The leading cause of death was lung cancer followed by stomach cancer. The average period from blood donation to death was 4.7 years (±2.3 years). Mean value and standard deviation of sFas among controls were 2.41 ± 1.81 ng/ml. sFas levels of the major cancer sites and control were shown in Figure 1. Cancer cases of each site shown here indicated higher mean values of sFas compared with controls, especially among cases of liver cancer or colon cancer.

Table II shows the distribution of selected characteristics of cases and controls. Gender and age were matching factors, and the distributions were almost equal. More smokers were observed among cases than controls ($p < 0.001$). The proportion of current drinkers and the distribution of BMI were not statistically different among cases and controls.

The risk of total cancer mortality increased with increasing sFas levels (trend $p = 0.005$), with OR of the highest quartile compared with the lowest being 1.96 (95% CI = 1.52–2.52) (Table III). This positive association remained unaltered after adjustment for covariates (OR = 1.81, 95% CI = 1.40–2.34). In analyses stratified by observation period, we found a 1.97-fold increased risk of cancer mortality (95% CI = 1.39–2.80) during the period from baseline to 1994, and a 1.93-fold increased risk (95% CI = 1.34–2.77) thereafter.

Discussion

This is the first report that has compared sFas levels among potential cancer cases from all sites and controls in a large-scale prospective study. We found that a higher sFas level was associated with an increased risk for cancer mortality, even among individuals whose cancer death occurred in the latter period (6.4 ± 1.4 years on an average after blood donation).

There have been several cross-sectional studies that compared sFas levels between cancer cases and healthy volunteers. Compared with those of controls, sFas levels are higher in the serum of cases with melanoma,[5] non-Hodgkin's lymphoma,[7] ovarian cancer,[3,12] cervical and endometrial cancer,[12] breast cancer,[2] hepatocellular carcinoma,[13] renal cell carcinoma[4] and bladder cancer.[1] Among cancer cases, some studies revealed sFas levels showed correlations with disease survival.[1-3,5,7,12] It was also confirmed in several studies that surgical removal of tumors reduced sFas levels.[4,13,14] As Holdenrieder and Stieber described in their review,[15] cancer itself has some self-defense mechanisms for avoiding apoptosis, one of which must be sFas.

Using stored serum from the participants of a large-scale cohort study before cancer diagnosis, we found a clear association between sFas levels and cancer mortality. This finding suggested that sFas could not only be a prognostic factor in patients with cancer but also a valid biomarker to identify people at high risk for cancer prior to diagnosis. Moreover, it is likely that cancer progression might be expedited and its prognosis worsened if cancer occurs in those with high serum sFas levels, because the risk elevation was almost the same in each observation period. Although the biological mechanisms that might explain the findings of this study have not been clarified yet, there is a possibility that cancer cells release sFas to avoid apoptosis even at the very early stage. However, from a nested case–control study in which cases and controls were selected from 3 cohort studies, Akhmedkhanov *et al.* failed to find any association between serum sFas drawn an average of 5.1 years before diagnosis and the incidence of ovarian cancer.[8] This contrast to the result from our study might be due to differences in the treated outcome [incidence and mortality, and ovarian cancer and cancer of all sites].

Our study has several strengths. We examined the associations between sFas levels and the risk of total cancer mortality in a prospective, nested case–control study. Sera were collected at an average of 4.7 years before cancer mortality. The prospective design could avoid the problems of questionable temporal relationships between sFas levels and cancer risks that hamper traditional case–control studies. In addition, a large number of cases were accumulated during the long follow-up period. Data on confounding factors were available, and their potential effects could be controlled by using multivariable analyses.

We also have to consider some limitations of our study when interpreting the results. First, since not all the cohort participants provided blood samples, the possibility of a selection bias could not be excluded. However, at the time of blood donation, no one could anticipate the subsequent cancer occurrence or mortality. Since cases and controls underwent the same process of selection before blood donation, that donation depended solely on the subject's intention. Thus, any bias due to blood donation or selection of cases and controls would not seriously affect our results. Second, serum samples were stored for ~10 years in deep freezers at −80°C. The stability of sFas in these cohort samples could not be determined because their values were not measured at baseline. However, Ito *et al.* compared newly collected sera and frozen specimens stored for 9 years, gathered from a variety of different individuals, and found no statistically significant difference in the distributions of sFas values,[11] indicating that the serum sFas level remained stable after long-term storage at −80°C. Furthermore,

TABLE II – CASE AND CONTROL DISTRIBUTION OF SOME DEMOGRAPHIC FACTORS

	Controls	Cases
Total number	2,353	798
Gender		
Men (%)	57.8	57.6
p-value	0.87	
Age at baseline		
40–49 (%)	5.1	5.4
50–59 (%)	20.9	20.6
60–69 (%)	46.2	45.6
70–79 (%)	27.8	28.4
Mean	64.2	64.3
SD	8.0	8.1
p-value*	0.90	
Smoking habits		
Current smoker (%)	27.0	37.2
p-value**	<0.001	
Drinking status		
Current drinker (%)	50.8	47.6
p-value**	0.08	
Body mass index		
<18.5 (%)	5.9	8.2
18.5<=, <25 (%)	75.7	72.1
25<= (%)	18.4	19.7
Mean	22.6	22.6
SD	3.1	3.0
p-value**	0.73	

p, performed by Mantel Haenszel test adjusted for area and age category.
**p*, performed by Mantel Haenszel test adjusted for area and gender.
***p*, performed by Mantel Haenszel test adjusted for area, gender and age category.

TABLE III – ODDS RATIOS OF CANCER MORTALITY ACCORDING TO sFAS LEVEL

	Control	Case	OR1	95% CI	Trend *p*	OR2	95% CI	Trend *p*
Total								
Q1	579	151	1.00			1.00		
Q2	627	188	1.20	0.94–1.54		1.17	0.91–1.51	
Q3	534	179	1.39	1.08–1.80		1.32	1.02–1.71	
Q4	613	280	1.96	1.52–2.52	0.005	1.81	1.40–2.34	0.007
Before 1994								
Q1	293	78	1.00			1.00		
Q2	319	95	1.19	0.84–1.67		1.19	0.84–1.68	
Q3	284	88	1.27	0.89–1.83		1.21	0.83–1.74	
Q4	342	159	1.97	1.39–2.80	0.031	1.81	1.26–2.58	0.042
After 1994								
Q1	286	73	1.00			1.00		
Q2	308	93	1.22	0.85–1.74		1.16	0.81–1.67	
Q3	250	91	1.53	1.06–2.21		1.44	0.99–2.09	
Q4	271	122	1.93	1.34–2.77	0.056	1.81	1.25–2.62	0.053

OR1, adjusted for age.
OR2, adjusted for age, BMI, smoking and drinking status.
sFas level: Q1 < 1.9; Q2, 1.9–2.2; Q3, 2.3–2.6; Q4, 2.7<= ng/ml.

the average 8.2% difference in sFas values between sera collected from 100 individuals in 1991 and in 1999 reported by Ito *et al.*[11] was similar to the coefficients of variation for determinations: 2.1–5.5% for intraassay and 8.2–12.3% for interassay. Third, although we have made some adjustment for possible confounders, there were some diseases, such as autoimmune disease, that might influence the level of sFas.[16] Unfortunately, it was impossible to adjust for these factors because of lack of information, however, if such diseases occurred at random, the estimated OR might approach null. In conclusion, our results suggest the possibility that serum sFas could indeed be a predictive marker of latent cancer before its diagnosis, since it increased in serum from apparently healthy people drawn an average 4.7 years before mortality.

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References

1. Mizutani Y, Yoshida O, Bonavida B. Prognostic significance of soluble Fas in the serum of patients with bladder cancer. J Urology 1998;160:571–6.
2. Ueno T, Toi M, Tominaga T. Circulating soluble Fas concentration in breast cancer patients. Clin Cancer Res 1999;5:3529–33.
3. Hefler L, Mayerhofer K, Nardi A, Reinthaller A, Kainz C, Tempfer C. Serum soluble Fas levels in ovarian cancer. Obstet Gynecol 2000;96:65–9.
4. Nonomura N, Nishimura K, Ono Y, Fukui T, Harada Y, Takaha N, Takahara S, Okuyama A. Soluble Fas in serum from patients with renal cell carcinoma. Urology 2000;55:151–5.
5. Ugurel S, Rappl G, Tilgen W, Reinhold U. Increased soluble CD95 (sFas/CD95) serum level correlates with poor prognosis in melanoma patients. Clin Cancer Res 2001;7:1282–6.
6. Puiu L, Petrakou E, Apostolidou A, Athanassiadou A, Psiouri L, Papachatzopoulou A, Gorgoulis V, Kotsinas A, Tzoracoeleftherakis E, Maniatis G, Voutsinas G. Lack of Fas (APO-1/CD95) gene structural alterations or transcript variant ratio changes in breast cancer. Cancer Lett 2003;194:91–7.
7. Niitsu N, Sasaki K, Umeda M. A high serum soluble Fas/APO-1 level is associated with a poor outcome of aggressive non-Hodgkin's lymphoma. Leukemia 1999;13:1434–40.
8. Akhmedkhanov A, Lundin E, Guller S, Lukanova A, Micheli A, Ma Y, Afanasyeva Y, Zeleniuch-Jacquotte A, Krogh V, Lenner P, Muti P, Rinaldi S, et al. Circulating soluble Fas levels and risk of ovarian cancer. BMC Cancer 2003;3:33.
9. Ohno Y, Tamakoshi A, JACC Study Group. Japan collaboraetive cohort study for evaluation of cancer risk sponsored by Monbusho (JACC Study). J Epidemiol 2001;11:144–50.
10. Tamakoshi A, Yoshimura T, Inaba Y, Ito Y, Watanabe Y, Fukuda K, Iso H, JACC Study Group. Profile of the JACC Study. J Epidemiol 2005;15:S4–S8.
11. Ito Y, Nakachi K, Imai K, Hashimoto S, Watanabe Y, Inaba Y, Tamakoshi A, Yoshimura T, JACC Study Group. Stability of frozen serum levels of insulin-like growth factor-I, insulin-like growth factor-II, insulin-like growth factor binding protein-3, transforming growth factor beta, soluble Fas, and superoxide dismutase activity for the JACC study. J Epidemiol 2005;15:S67–S73.
12. Konno R, Takano T, Sato S, Yajima A. Serum soluble Fas level as a prognostic factor in patients with gynecological malignancies. Clin Cancer Res 2000;6:3576–80.
13. Jodo S, Kobayashi S, Nakajima Y, Matsunaga T, Nakayama N, Ogura N, Kayagaki N, Okumura K, Koike T. Elevated serum levels of soluble Fas/APO-1 (CD95) in patients with hepatocellular carcinoma. Clin Exp Immunol 1998;112:166–71.
14. Pignataro L, Arisi E, Sambataro G, Corsi MM. Soluble Fas (sFas) and soluble Fas ligand (sFas-L) balance in laryngeal carcinoma before and after surgical treatment. J Surg Oncol 2003;83:112–5.
15. Holdenrieder S, Stieber P. Apoptotic markers in cancer. Clin Biochem 2004;37:605–17.
16. Feldkamp J, Pascher E, Schott M, Goretzki P, Seissler J, Scherbaum WA. Soluble Fas is increased in hyperthyroidism independent of the underlying thyroid disease. J Clin Endocrinol Metab 2001;86:4250–3.

Exon 19 of *EGFR* mutation in relation to the CA-repeat polymorphism in intron 1

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Epidermal growth factor receptor (*EGFR*) mutations in lung cancer enhance tyrosine kinase activity and increase sensitivity to the EGFR tyrosine kinase inhibitor, gefitinib. Mutation analysis of the *EGFR* gene is therefore indispensable for predicting gefitinib response. We investigated a CA-repeat polymorphism in the *EGFR* gene related to *EGFR* mutations. Because an increasing number of CA-repeats at intron 1 of the *EGFR* gene has been reported to reduce transcription activity, we examined the relationship between *EGFR* mutations and this CA-repeat polymorphism. *EGFR* mutations at exon 19 were closely associated with shorter CA-repeat length in the shorter allele, but this was not the case for *EGFR* mutations at exons 18 or 21. Increased intrinsic *EGFR* mRNA expression in non-cancerous lung tissues from lung adenocarcinoma patients was also significantly associated with shorter CA-repeat length. A higher frequency of *EGFR* mutations at exon 19 was associated with shorter CA-repeat length only in patients with high levels of *EGFR* mRNA expression. To determine the phenotypes of cells possessing shorter CA-repeats, an *in vitro* study using human bronchial epithelial cells with different CA-repeat lengths was performed; more rapid cell growth and activated EGF/EGFR signaling were found more often in the cells having both shorter CA-repeats and increased *EGFR* mRNA expression. These results suggest that CA-repeat length in the *EGFR* gene may be a genetic factor related to cancer in the case of *EGFR* mutations at exon 19. The mechanism likely involves enhanced intrinsic expression of *EGFR* mRNA and activated EGF/EGFR signaling that accompany shorter CA-repeats. (*Cancer Sci* 2008; 99: 1180–1187)

Lung cancer is the most common form of cancer death among males and the third most common among females in Japan.[1] Overall survival from lung cancer remains unsatisfactory, with a five-year survival rate of 14%, indicating that this is one of the most difficult cancers to treat.[2] However, the therapeutic strategy has changed since molecular targeting therapies, such as the use of epidermal growth factor receptor (EGFR) tyrosine kinase inhibitors, became available. Specifically, mutations in the *EGFR* gene found in lung cancer tissues have been reported to be a predictive marker for clinical response to EGFR tyrosine kinase inhibitor therapy, which may lead to the development of customized therapy.[3–6]

Mutations in the *EGFR* gene were found in 30–40% of all lung cancer patients in Japan, and were found in over 50% of female non-smokers with adenocarcinomas.[7–10] Although the overall rate of response to EGFR tyrosine kinase inhibitors for previously treated patients was 27.5% among Japanese,[11] the frequency of gefitinib responsive patients among those with *EGFR* mutations was approximately 80%.[5,9] Therefore, it is clear that DNA sequence analysis of *EGFR* in lung cancer is essential for predicting response to EGFR tyrosine kinase inhibitor therapy. In addition, *EGFR* mutations have been reported to be involved in lung carcinogenesis; an investigation using transgenic mice carrying the *EGFR* mutants $EGFR^{L858R}$ and $EGFR^{\Delta L747-S752}$ demonstrated that mutant *EGFR* was required for the development and maintenance of lung adenocarcinomas.[12] Identification of clinical and genetic factors underlying *EGFR* mutations will be particularly meaningful not only for understanding lung carcinogenesis in non-smokers but also for making clinical decisions about the use of EGFR tyrosine kinase inhibitors in non-operative patients.

The *EGFR* gene is located on chromosome 7p12.1–12.3 in humans, and its expression is regulated by one promoter region and two enhancer regions.[13] The promoter region contains a GC-rich sequence without the characteristic TATA and CAAT boxes, and multiple transcription start sites exist. At least four Sp1 binding sites and one TC factor binding site are known, and basal transcription is regulated by Sp1.[14,15] Two enhancer elements, one located upstream of the promoter (–1429/–1109) and one located downstream at intron 1 (+1788/+2318), function cooperatively.[16] A polymorphic simple sequence repeat with 14–21 CA-repeats was first demonstrated close to the downstream enhancer by Chi *et al.*[17] An increased number of CA-repeats was reported to be associated with decreased *EGFR* transcription activity: a 2-fold increase in transcription activity with 16 CA-repeats compared to that with more than 18 CA-repeats.[18] An *in vitro* run-off assay using a 4050 bp polymerase chain reaction (PCR) product of the *EGFR* gene showed that the level of *EGFR* transcription was reduced by 80% with 21 CA-repeats compared to that with 16 CA-repeats. Using competitive reverse transcription-polymerase chain reaction (RT-PCR), it was demonstrated that pre mRNA expression levels in various cancer cell lines were correlated with the number of CA-repeats; higher levels of *EGFR* mRNA were found in cancer cells with lower numbers of CA-repeats, which is consistent with the *in vitro* experiments.

The relationship between lung cancer and CA-repeat polymorphism has been studied in terms of lung cancer risk or clinical phenotype, specifically gefitinib responsiveness. A case-control study revealed an inverse relationship between lung cancer risk and the number of CA-repeats in a Caucasian population,[19] although no association with lung cancer risk was reported in a Korean population.[20] A clinical study of 86 patients with advanced non-small-cell lung cancer treated with gefitinib in Korea showed that a low number of CA-repeats was associated with gefinitib responsiveness, although no significant association with *EGFR* mutation status nor *EGFR* expression levels was found in cancer tissues.[21] On the other hand, it has been reported that shorter CA-repeat length is associated with an increased expression of *EGFR*.[18,22–24] Furthermore, shorter CA-repeat alleles in lung cancer were more likely to be amplified, resulting in more prevalent allelic imbalance at the *EGFR* locus; *EGFR* mutations were found to favor shorter CA-repeat alleles.[25] However, most of these studies were based on cancer cell lines and resected cancer tissues.

We previously reported that *EGFR* mutations at exons 18, 19 and 21 evidenced different clinical profiles, suggesting that

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EGFR mutations should be analyzed according to the exons at which they occur.[10] In addition, we think that the relationship between CA-repeat polymorphism and intrinsic *EGFR* expression should also be examined in non-cancerous tissues and cultured normal lung epithelial cells, because *EGFR* expression in cancer tissues may be modified by various genetic alterations such as mutations and allelic imbalance. Indeed, in lung cancer tissues with the same CA-repeat status, *EGFR* mRNA levels differed according to whether the *EGFR* allele was wild-type or mutated, and also depended on the *EGFR* copy number.[26]

In the present study, seeking to identify the genetic factors underlying *EGFR* mutations, we investigated the relationship between the CA-repeat polymorphism and *EGFR* mutations according to exon in 154 patients with lung cancer, 70% of which were of pathological stages I and II. We also studied the association between CA-repeat polymorphism and *EGFR* mRNA levels in non-cancerous tissues from 74 lung adenocarcinoma patients being followed in an *in vitro* study of CA-repeat length, and cell growth using normal human bronchial epithelial (HBE) cells with different CA-repeat lengths obtained from 11 lung cancer patients.

Materials and Methods

Tissue and pleural effusion. We studied a total of 154 Japanese patients with lung cancer. We obtained tissue specimens as follows: 123 surgical specimens, 9 transbronchial fiberscopic specimens, 19 pleural effusions from non-resectable lung cancers, two specimens from metastatic lesions of the brain and skin, and one sample of cells from urine. Study patients were admitted to the Saga Medical School Hospital, Saga, Japan, between 2000 and 2007; 142 patients had not received anticancer chemotherapy or thoracic irradiation, and 12 patients were recurrent cases. Clinical stage was determined according to the criteria of the International Union Against Cancer. Histological subtype and tumor content were confirmed by Hematoxylin and Eosin staining with tumor samples; pleural effusion was assessed as class V by a pathologist. *EGFR* mutation status and CA-repeat length were investigated using DNA direct sequencing. All procedures were performed with the informed consent of the patients, and the study was approved by the Saga University Institutional Review Board Committee.

DNA extraction and sequencing analysis. DNA was isolated from freshly frozen lung cancer tissues using a QIAamp DNA mini kit (QIAGEN, Hilden, Germany) following the manufacturer's instructions. Mutations at exons 18, 19, and 21 were determined using PCR-based direct sequencing in cancer tissues; numbers of CA-repeats were determined using adjacent, normal (non-cancerous) tissues. The primers used for *EGFR* mutations were previously described.[3,10] The primer sets used for determination of CA-repeat were 5′-CGGCTGTCCGGCCACTGG-3′ (sense) and 5′-CAGCTCAAGGTTGGAATTGTGC-3′ (antisense) (amplicon size: 378 bp). PCR amplification was performed in a 20-μL volume using Discoverase DHPLC DNA polymerase (Invitrogen Inc., CA, USA) at 95°C for 10 min followed by 40 cycles (each cycle at 95°C for 30 s, 58°C for 30 s, and 72°C for 1 min), with a final extension at 72°C for 10 min. The amplified products were isolated using Microcon YM-50 (Millipore Inc., MA, USA) and sequenced directly using the Applied Biosystems PRISM dye terminator cycle sequencing method with an ABI PRISM 310 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA).

***EGFR* mRNA expression.** Total RNAs were isolated from both lung cancer tissues and non-cancerous tissues using ISOGEN reagent (Nippon gene, Japan). Levels of *EGFR* mRNA were determined by real-time RT-PCR. One μg total RNA was applied to RT with MuLV reverse transcriptase (Roche Molecular Systems, NJ, USA) at 37°C for 60 min. The cDNAs obtained were processed by quantitative SYBR Green real-time PCR. Each 20 μL SYBR Green reaction consisted of 2 μL cDNA, 2 μL 10 × LightCycler-DNA Master SYBR Green I (Roche Diagnostics Corporation, IN), and 1 μM each of forward and reverse primers. *EGFR* specific primers were 5′-GTCTCTTGCCGGAATGTCAG-3′ (sense) and 5′-CTCACCCTCCAGAAGGTTGC-3′ (antisense) (amplicon size: 67 bp), as previously reported.[27] Quantitative PCR was performed on a Light-Cycler V3 System (Roche Diagnostics Corporation, IN, USA) with 60 cycles, using three-stage program parameters for each cycle as recommended by the manufacturer: 2 s at 95°C, 10 s at 62°C, and 15 s at 72°C. A melting curve analysis was run to assess specificity of the amplified PCR products. Quantification focused on the initial exponential phase of amplification above baseline according to the Light-Cycler software. *EGFR* mRNA levels were standardized by *β-actin* mRNA and log-transformed as log(*EGFR* mRNA/*β-actin* mRNA) for both cancer and non-cancerous tissues.

Cell culture and assessment of cell growth. Primary HBE cells were isolated from bronchial mucosal biopsies of eight lung cancer patients with CA 16/15 (repeat number of the longer allele/shorter allele), six patients with CA 20/19, and one patient with CA 8/7. Following isolation, cells were cultured in Keratinocyte Serum-Free Medium® (Gibco BRL, Life Technologies, Inc., Rockville, MD, USA) containing EGF and bovine pituitary extract at 37°C in 5% CO_2 as described previously.[28] Cells were subjected to experiments after three passages. *EGFR* mRNA detection was performed in the same way as described above for tissue samples.

Western blot analysis. Normal HBE cells were first incubated for 24 h in medium without EGF and bovine pituitary extract, then treated with 100 ng/mL EGF (Sigma, Saint Lous, MS, USA). Whole cell lysates were prepared from cells using lysis buffer containing 50 mM Tris-HCL pH 8.0, 150 mM NaCl, 5 mM $MgCl_2$, 1% TritonX-100, 0.1% sodium dodecyl sulfate, 0.5% sodium deoxycholate, 40 mM sodium fluoride, 1 mM sodium orthovanadate, 1 μg/mL leupeptin, 10 μg/mL aprotinin, and 1 mM phenolmethylsulfonyl fluoride, as reported previously.[12] Protein (50 μg) was separated using a 10% NuPAGE electrophoresis system (NOVEX, San Diego, CA, USA), transferred to a nitrocellulose membrane (Schleicher & Schuell, Inc., Keene, NH, USA), blocked with 5% milk at 4°C overnight, and finally reacted with anti-EGFR, Erk1/2 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA, antiphospho EGFR, or antiphospho Erk1/2 antibodies (Cell Signaling Technology, Inc., Danvers, MA, USA). An ECL kit (Amersham Corp., Arlington Heights, IL, USA) was used for detection.

Statistical analysis. Baseline characteristics of patients with or without *EGFR* mutations were compared using the χ^2 test for categorical data and the Student's *t*-test for continuous data. A logistic regression model was used to evaluate the contribution of CA-repeat to *EGFR* mutations among lung adenocarcinoma patients, considering gender, age, and smoking status as potential confounders. Differences in means of log(*EGFR* mRNA/*β-actin* mRNA) between two groups defined by CA-repeat number (less than 17 vs 17 or more) in the shorter allele were compared using the Student's *t*-test. The effect of CA-repeat length on *EGFR* mRNA expression level was evaluated by categorical regression analysis with optimal scaling using alternation least squares with adjustment for age, gender, and smoking status (SPSS Categories version 11.0, SPSS Inc., Chicago, IL, USA). For this analysis, we categorized the dependent variable into seven ranks with an approximately normal distribution: log(*EGFR* mRNA/*β-actin* mRNA) ≤–2.23, –1.61–0.12, 0.06–1.37, 1.41–2.58, 2.68–3.47, 4.12–5.40, and ≥5.75. Association between CA-repeat length and *EGFR* mutations at exon 19 was assessed using the $f\bar{O}^{(2)}$ test separately by level of log(*EGFR* mRNA/*β-actin* mRNA), which was classified into two groups according to the median (<2.10, ≥2.10). Cell growth was compared between the two groups of CA-repeat length in normal HBE cells using the Kruskal–Wallis test; doubling times and *EGFR* mRNA levels were compared using the Mann–Whitney test.