Introduction

Cancer cells adapt in response to the pressure exerted upon them by various hormonal treatments. Ultimately, this process of adaptation renders them insensitive to hormonal therapy. In patients, clinical observations suggest that long term deprivation of estradiol causes breast cancer cells to develop enhanced sensitivity to the proliferative effects of estrogen. Premenopausal women with advanced hormone dependent breast cancer experience objective tumor regressions in response to surgical oophorectomy which lowers estradiol levels from mean levels of approximately 200 pg/ml to 10 pg/ml.[1] After 12–18 months on average, tumors begin to regrow even though estradiol levels remain at 10 pg/ml. Notably tumors again regress upon secondary therapy with aromatase inhibitors which lower estradiol levels to 1–2 pg/ml. These observations suggest that tumors develop hypersensitivity to estradiol as demonstrated by the fact that untreated tumors require 200 pg/ml of estradiol to grow whereas tumors regrowing after oophorectomy require only 10 pg/ml. We have shown in prior studies that up-regulation of growth factor pathways contributes to the phenomenon of hypersensitivity.[2–10] Ultimately these tumors adapt further and grow exclusively in response to growth factor pathways and do not require estrogens for growth.

In order to provide direct proof that hypersensitivity does develop and to study the mechanisms involved, we have utilized cell culture and xenograft models of breast cancer as experimental tools.[5,8,9,11–13]

Phenomenon of Hypersensitivity: Mechanisms and Pathways

To induce hypersensitivity, wild type MCF-7 cells require culturing over a 6–24 month period in estrogen-free media to mimic the effects of ablative endocrine therapy such as induced by surgical oophorectomy or aromatase inhibitors.[11,12] is process involves Long Term Estradiol Deprivation and the adapted cells are called by the acronym, LTED cells. As evidence of hypersensitivity, a three log lower concentration of estradiol can stimulate proliferation of LTED cells compared to wild type MCF-7 cells (Fig. 1A).[7] We reasoned that the development of hypersensitivity could involve modulation of the genomic effects of estradiol acting on transcription, nongenomic actions involving plasma membrane related receptors, cross talk between growth factor and steroid hormone stimulated pathways, or interactions among these various effects.[5,7–9,11–13]

We initially postulated that enhanced receptor mediated transcription of genes related to cell proliferation might be involved. Indeed, the levels of ERα increased 4–10 fold during long term estradiol deprivation.[11] The up-regulation of ER alpha results from demethylation of promoter A and C of the estrogen receptor (Fig. 1B and 1C). The transcripts stimulated by this promoter increase by 149 fold and the DNA of this segment exhibits a marked increase in demethylation. [13A]We initially reasoned that the up-regulation of ERα would directly result in hypersensitivity to estradiol (E_2). Accordingly, to directly examine whether enhanced sensitivity to E_2 in LTED cells occurred at the level of ER mediated transcription, we quantitated the effects of estradiol on transcription in LTED and in wild type MCF-7 cells. As transcriptional readouts, we measured the effect of E_2 on progesterone receptor (PgR) and pS2 protein concentrations and on ERE-CAT reporter activity (Fig. 2A–F).[9,13] We observed no shift to the left in estradiol dose response curves (the end point utilized to detect hypersensitivity) for any of these responses (i.e., PgR, pS2, CAT activity) when comparing LTED with wild type MCF-7 cells. On the other hand, basal levels (i.e., no estrogen added) of transcription of three ER/ERE related reporter genes were greater in LTED than in wild type MCF-7 cells (Fig. 2D–F).[13]

To interpret these data, we used the classic definition for hypersensitivity, namely a significant shift to the left in the dose causing 50% of maximal stimulation. Accordingly, these data suggest

that hypersensitivity of LTED cells to the proliferative effects of estradiol does not occur primarily at the level of ER-mediated gene transcription (Fig. 2A–C) but may be influenced by the higher rates of maximal transcription (Fig. 2D–F).

We next considered that adaptation might involve dynamic interactions between pathways utilizing steroid hormones and those involving MAP kinase and PI-3-kinase for growth factor signaling (Fig. 3A).[5,7–9,11–16] Our initial approach demonstrated that basal levels of MAP kinase were elevated in LTED cells in vitro (Fig. 2B, top panel) and in xenografts (data not shown) and were inhibited by the pure antiestrogen, fulvestrant.[8,11]

We further demonstrated that activated MAP kinase is implicated in the enhanced growth of LTED cells since inhibitors of MAP kinase such as PD98059 or U-0126 block the incorporation of tritiated thymidine into DNA.[7] To demonstrate proof of the principle of MAP kinase participation, we stimulated activation of MAP kinase in wild type MCF-7 cells by administering TGFα (data not shown). Administration of TGFα caused a two log shift to the left in the ability of estradiol to stimulate the growth of wild type MCF-7 cells. To demonstrate that this effect was related specifically to MAP kinase and not to a nonMAP kinase mediated effect of TGF alpha, we co-administered PD 98059. Under these circumstances, the two log left shift in estradiol dose response, returned back to the baseline dose response curve.[7] As further evidence of the role of MAP kinase, we administered U-0126 to LTED cells and examined its effect on level of sensitivity to estradiol. is agent partially shifted dose response curves to the right by approximately one-half log (data not shown).

While an important component, MAP kinase did not appear to be solely responsible for hypersensitivity to estradiol. Blockade of this enzyme did not completely abrogate hypersensitivity. Accordingly, we examined the PI-3-kinase pathway to determine if it was up-regulated in LTED cells as well (Fig. 3B) and examined several signaling molecules downstream from this regulatory kinase.[16] We determined that LTED cells exhibit an enhanced activation of AKT (Fig. 3B, second panel), P70 S6 kinase (Fig. 3B, third panel) and PHAS-1/4E BP-1 (Fig. 3B, fourth panel; see also below).[16] Dual inhibition of PI-3-kinase with Ly 294002 (specific PI-3-kinase inhibitor) and MAP kinase with U-0126 shifted the level of sensitivity to estradiol more dramatically: more than two logs to the right (Fig. 3C).[7]

One possible mechanism to explain the activation of MAP kinase would be through nongenomic effects of estrogen acting via ERα located in or near the cell membrane.[17–19] We postulated that membrane associated ERα might utilize a classical growth factor pathway to transduce its effects in LTED cells. The adaptor protein SHC represents a key modulator of tyrosine kinase activated peptide hormone receptors.[14–15,20] Upon receptor activation and auto-phosphorylation, SHC binds rapidly to specific phosphotyrosine residues of receptors through its PTB or SH2 domain and becomes phosphorylated itself on tyrosine residues of the CH domain.[14,15] The phosphorylated tyrosine residues on the CH domain provide the docking sites for the binding of the SH2 domain of Grb2 and hence recruit SOS, a guanine nucleotide exchange protein. Formation of this adapter complex allows Ras activation via SOS, leading to the activation of the MAPK pathway.[20]

We postulated that estrogen deprivation might trigger activation of a nongenomic, estrogen-regulated, MAP kinase pathway which utilizes SHC.[14–15,20–22] We employed MAP kinase activation as an endpoint with which to demonstrate rapid nongenomic effects of estradiol (Fig. 4A). The addition of E_2 stimulated MAP kinase phosphorylation in LTED cells within minutes. The increased MAP kinase phosphorylation by E_2 was time and dose-dependent, being greatly stimulated at 15 min and remaining elevated for at least 30 min. Maximal stimulation of MAP kinase phosphorylation was at 10^{-10} M of E_2.

We then examined the role of peptides known to be involved in growth factor signaling pathways that activate MAP kinase. SHC proteins are known to couple tyrosine kinase receptors to the MAPK pathway and activation of SHC involves the phosphorylation of SHC itself.[20–22] To investigate if the SHC pathway was involved in the rapid action of estradiol in LTED cells, we immunoprecipitated tyrosine phosphorylated proteins and tested for the presence of SHC under E_2 treatment. E_2 rapidly stimulated SHC tyrosine phosphorylation in a dose and time dependent fashion with a peak at 3 minutes.[20] The pure estrogen receptor antagonist, fulvestrant, blocked E_2-induced SHC and MAPK phosphorylation at 3 min and 15 min respectively. To demonstrate that the classical ER alpha mediated this response, we transfected a siRNA against ER alpha and showed down-regulation of this receptor and also abrogated the effect of estradiol to rapidly enhance MAP kinase activation. The time frame suggests that SHC is an upstream component in E_2-induced MAPK activation.

We reasoned that the adapter protein SHC may directly or indirectly associate with ERα in LTED cells and thereby mediate E_2-induced activation of MAP kinase. We considered this likely in light of recent evidence regarding ERα membrane localization.[23–25] To test this hypothesis, we immunoprecipitated SHC from nonstimulated and E_2-stimulated LTED cells and then probed immunoblots with anti-ERα antibodies. Our data showed that the ERα/SHC complex pre-existed before E_2 treatment and E_2 time-dependently increased this association.[20] In parallel with SHC phosphorylation, we observed a maximally induced association between ERα and SHC at 3 min (data not shown). MAP kinase pathway activation by SHC requires SHC association with the adapter protein Grb2 and then further association with SOS. By immunoprecipitation of Grb2 and detection of both SHC and SOS, we demonstrated that the SHC-Grb2-SOS complex constitutively existed at relatively low levels in LTED cells, but was greatly increased by treatment of cells with 10^{-10} M E_2 for 3 min.[20]

After the demonstration of protein-protein interactions, we wished to provide evidence that these biochemical steps resulted in biologic effects. Accordingly, we evaluated the role of estrogen activated MAP kinase on the function of the transcription factor, Elk-1. When activated, Elk-1 serves as a down stream mediator of cell proliferation. The phosphorylation of Elk1 by MAPK can up-regulate its transcriptional activity through phosphorylation. By cotransfection of LTED cells with both GAL4-Elk and its reporter gene GAL4-luc,[26,27] we were able to show that E_2 dose-dependently increased Elk-1 activation at 6 hours as shown by luciferase assay (Fig. 4B).[20]

We also wished to demonstrate biologic effects on cell morphology. To examine E_2 effects on reorganization of the actin cytoskeleton, we visualized the distribution of F-actin by phalloidin staining and also redistribution of the ERα localization in LTED and MCF-7 cells (data not shown).[20] Untreated MCF-7 cells expressed low actin polymerization and a few focal adhesion points. After E_2 stimulation, in contrast, the cytoskeleton underwent remodeling associated with formation of cellular ruffles, lamellipodia and leading edges, alterations of cell shape and loss of mature focal adhesion points. A sub-cellular redistribution of ERα to these dynamic membranes upon E_2 stimulation represented another important feature. The ER antagonist ICI 182 780 at 10^{-9} M blocked E_2-induced ruffle formation as well as redistribution of ERα to the membrane with little effect by itself. Therefore, these studies further demonstrated the rapid action of E2 with respect to dynamic membrane alterations in LTED cells.

A key unanswered question was how the ER could localize in the plasma membrane when it does not contain membrane localization motifs. We postulated that the IGF-1-receptor and SHC might be involved in this process (Fig. 5A).[28] A series of studies by other investigators suggested that ERα and the IGF-1 receptor might interact.[28] We tested the model that estradiol caused binding of SHC to ERα but also caused phosphorylation of the IGF-1 receptor. In this way, SHCwould serve as the "glue" which would tether ER alpha to the plasma membrane

where it would bind to the SHC acceptor site. To assess this possibility, we immunoprecipitated IGF-1 receptors before and after addition of estradiol. is caused SHC to bind to the IFG-1 receptor (Fig. 5C) and caused the IGF-1 receptor to become phosphorylated (Fig. 5B,C). In order to prove a causal effect for this role of SHC, we utilized an siRNA methodology to knock down SHC and showed that this prevented ERα from binding to the IGF-1 receptor.[28] As further evidence, we conducted confocal microscopy experiments to show that knockdown of SHC prevented ERα from localizing in the plasma membrane (data not shown).[29]

Ellis Levin and colleagues recently showed that ER alpha must be palmitylated before it can localize in the plasma membrane.[29A] Although speculative, we postulate that ER alpha requires palmitylation to travel to the plasma membrane but activated SHC serves to tether it to the membrane via IGF-1-R. In contrast to our previous concept that SHC serves as the "bus" to carry ER alpha to the membrane, we now postulate that SHC is the "glue" that tethers ER alpha there after binding to the IGF-1-R. Further studies will be necessary to dissect out each component of these interactions and their biologic relevance.

From the data reviewed, we conclude that membrane related ERα plays a role in cell proliferation and in activation of MAP kinase. It appeared likely then that LTED cells might exhibit enhanced functionality of the membrane ERα system. As evidence of this, we examined the ability of estradiol to cause the phosphorylation of SHC in wild type and MCF-7 cells and also to cause association of SHC with the membrane ERα. We demonstrated a marked enhancement of both of these processes in LTED as opposed to wild type cells. Considering all of these data together, it is still not clear at the present time what is responsible for enhancement of the nongenomic ERα mediated process.

If adaptive hypersensitivity results from the up-regulation of growth factor pathways, an inhibitor of MAP kinase and downstream PI-3-kinase pathways could be important in abolishing hypersensitivity and in inhibiting cell proliferation. We had been studying the effects of a MAP kinase inhibitor, farnesylthiosalicylic acid (FTS), which has been shown to block proliferation of LTED cells. is agent interferes with the binding of GTP-Ras to its acceptor site in the plasma membrane, a protein called galectin 1.[30] While examining its downstream effects, we have shown that this agent is also a potent inhibitor of phosphoinositol-3-kinase (PI-3-kinase). We postulated that an agent which blocks not only the MAP kinase pathway but also downstream actions of the PI-3-kinase pathway might be ideal to inhibit hypersensitivity. Accordingly, we have intensively studied the effects of FTS on mTOR.

The mammalian target of rapamycin, mTOR, is a Ser/r protein kinase involved in the control of cell growth and proliferation.[31] One of the best characterized substrates of mTOR is PHAS-1 (also called 4E-BP1).[32,33] PHAS-I/4E-BP1 binds to eIF4E and represses cap-dependent translation by preventing eIF4E from binding to eIF4G.[32,33] When phosphorylated by mTOR, PHAS-I/4E-BP1 dissociates from eIF4E, allowing eIF4E to engage eIF4G, thus increasing the formation of the eIF4F complex needed for the proper positioning of the 40S ribosomal subunit and for efficient scanning of the 5'-UTR.[31] In cells, mTOR is found in mTORC1, a complex also containing raptor, a newly discovered protein of 150kDa. It has been proposed that raptor functions in TORC1 as a substrate-binding subunit which presents PHAS-I/4E-BP1 to mTOR for phosphorylation.[31,32] Our results suggest that FTS inhibits phosphorylation of the mTOR effectors, PHAS-I/4E-BP1 and S6K1, in response to estrogen stimulation of breast cancer cells.[2]

To investigate the effects of FTS on mTOR function, we utilized 293T cells and monitored changes in the phosphorylation of PHAS-I/4E-BP1.[2] Incubating cells with increasing concentrations of FTS decreased the phosphorylation of PHAS-I/4E-BP1, as evidenced by a

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.

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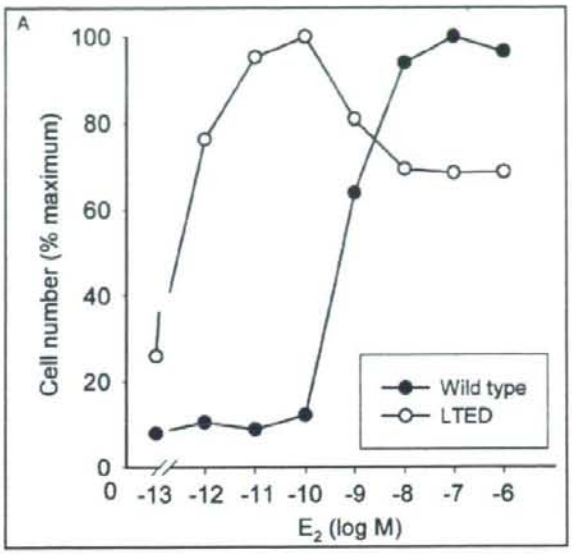

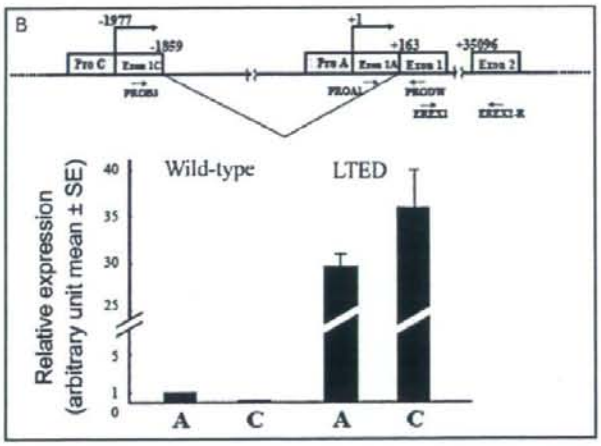

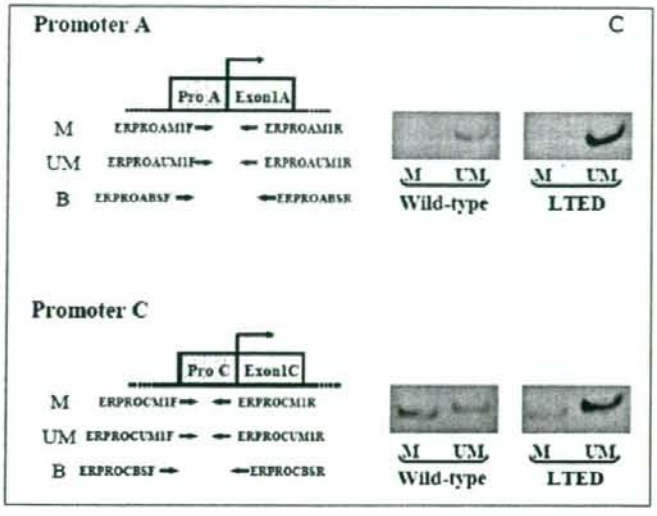

Figure 1.

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.

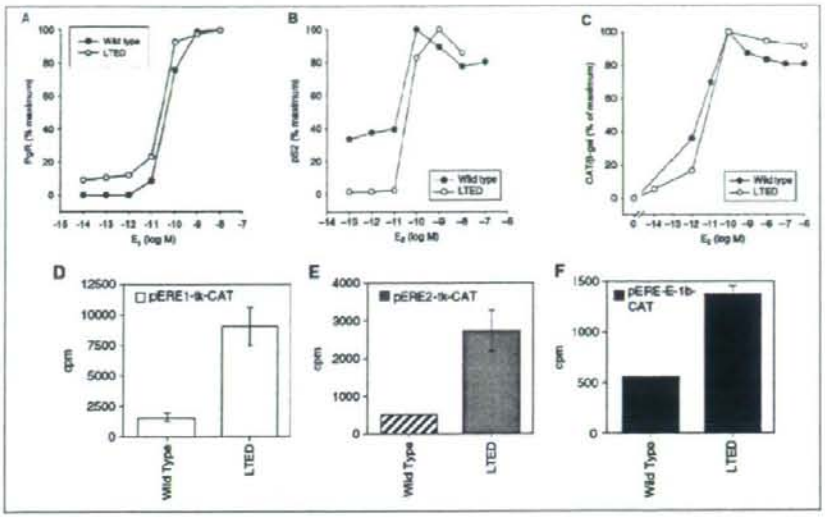

Figure 2.

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.

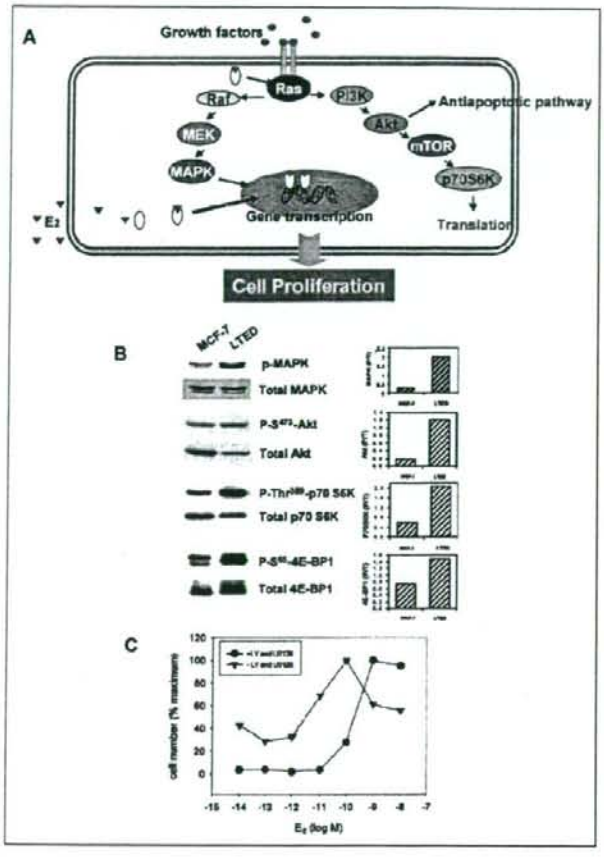

Figure 3.

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.

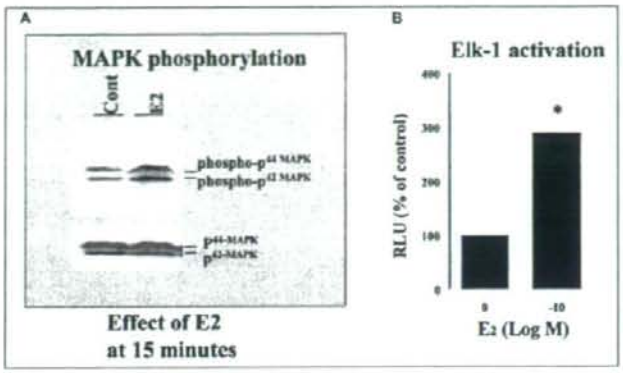

Figure 4.

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.

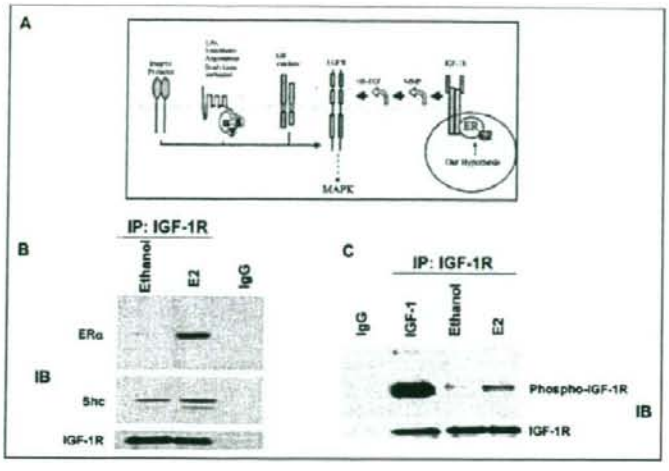

Figure 5.

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.

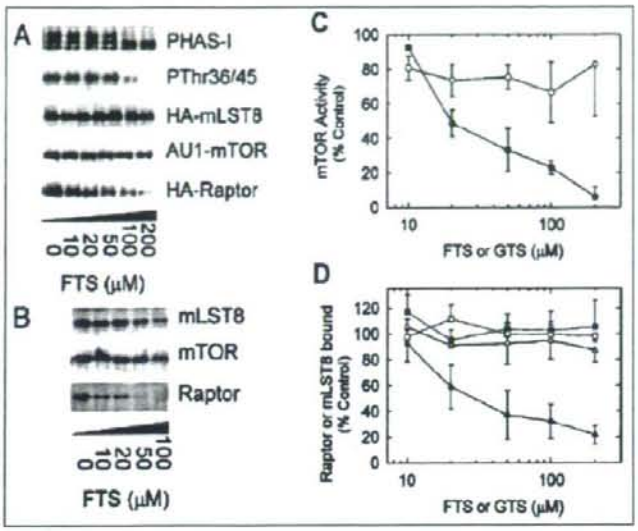

Figure 6.

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 (○, △, □) before immunopreciptations 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 (■, △) 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.

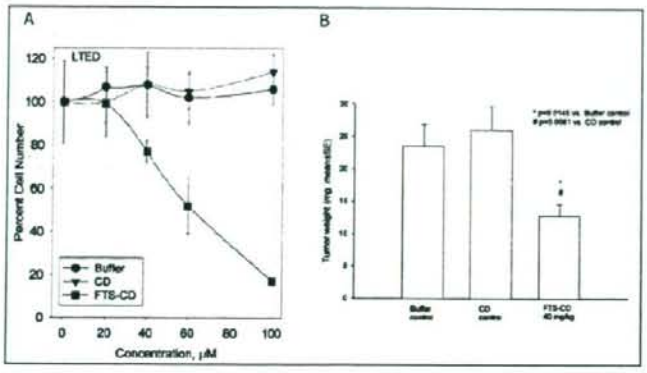

Figure 7.

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 $p = 0.0061$.

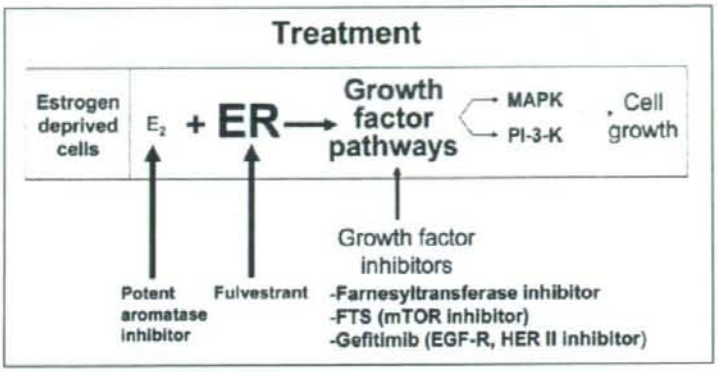

Figure 8.
Practical implications of the effects of up-regulation of growth factor pathways and development of hypersensitivity to estradiol. Potent aromatase inhibitors are useful to counteract the enhanced sensitivity to estradiol resulting from adaptation to prolonged estradiol deprivation. A pure 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

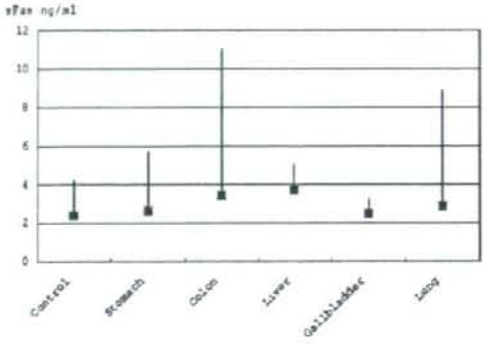

FIGURE 1 – Mean values and standard deviation of sFas among major sites of cancer and control.

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.