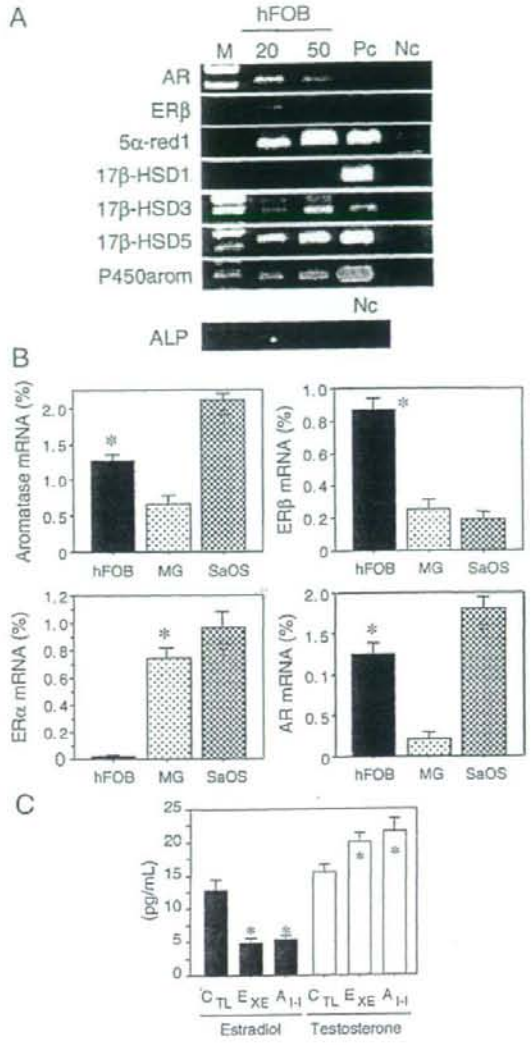

Fig. 2. (A) Results of RT-PCR analysis of steroid hormone receptors and steroid-related enzymes. Both 20 and 50 ng/μL cDNA of hFOB were used for PCR (ALP was 20 ng/μL alone). AR, androgen receptor; ER, estrogen receptor; 5α-red1, 5α-reductase type 1; 17β-HSD, 17β-hydroxysteroid dehydrogenase; P450 arom, aromatase; M, molecular marker; Pc, positive control; Nc, negative control. (B) Expression levels of aromatase, AR, ERα, and ERβ in hFOB, Saos-2, and MG-63. $*p<0.05$ vs. MG-63 (aromatase and AR), vs. MG-63 and vs. Saos-2 (ERβ), vs. hFOB (ERα); $^{\dagger}p<0.05$ vs. hFOB and MG-63 (aromatase and AR), vs. MG-63 and hFOB (ERα). (C) Estradiol and testosterone productions in hFOB cells. The data are expressed as the mean SD ($n=3$). $*p<0.05$ vs. control cells (CTL). EXE, 10^{-7} M exemestane; AI-I, 10^{-7} M aromatase inhibitor I.

using the *p*-nitrophenylphosphate method (LabAssay ALP; Wako Pure Chemical industries) [19]. Optical densities (OD, 405 nm) were evaluated using a SpectraMax 190 microplate reader (Molecular Devices) and Softmax Pro 4.3 microplate analysis software (Molecular Devices). ALP activity (units/μL)=(concentration of *p*-nitrophenol/15 min)×1 (dilution factor of sample). The ALP activities were presented as units/μL/10^6 cells. The ALP activity levels in each case were represented as a ratio of vehicle control (%).

Microarray analysis

The procedure was based on a previously reported study [20]. Cell lysates were prepared using RLT buffer (QIAGEN GmbH, Hilden, Germany). Total RNA was extracted using RNeasy Mini Kit (QIAGEN). First-strand cDNA was synthesized by incubating 5 μg of total RNA with 200 U SuperScript II reverse transcriptase (Invitrogen), 100 pmol T7-(dT)24 primer (Invitrogen). Ten units of T4 DNA polymerase (Invitrogen) were then added, and the dsDNA was mixed with T7 RNA polymerase (Invitrogen). The purified cRNA was fragmented at 300–500 bp as target solution. Hybridization was performed with the GeneChip Human Genome 133 ver. 2.0 (Affymetrix, Inc., CA, USA). The reacted arrays were then scanned as digital image files and scanned data were analyzed with GeneChip software (Affymetrix). Relative levels of gene expression were calculated by global normalization.

Data were subjected to hierarchical clustering analysis and visualization using the Cluster and TreeView programs (Stanford University) in order to generate tree structures based on the degree of similarity, as well as matrices comparing the levels of expression of individual genes in each sample [21].

Real-time PCR

Real-time PCR was carried out using the LightCycler System and the FastStart DNA Master SYBR Green I (Roche Diagnostics GmbH, Mannheim, Germany). The primer sequences used in this study are summarized in Table 2. An initial denaturing step of 95 °C for 10 min was followed by 35 cycles, respectively, at 95 °C for 10 min; 15 s annealing at 65 °C (ALP, COL1A1), 64 °C (MYBL2, OSTM1, RPL13A), 62 °C (SMAD1, SMAD5, SPARC, RUNX2), or 60 °C (HOXD11); and extension for 15 s at 72 °C. Negative control experiments included those lacking cDNA substrates to confirm the presence of exogenous contaminant DNA. No amplified products were detected under these conditions. The mRNA levels in each case were represented as a ratio of RPL13A (%) [22].

Immunohistochemistry of AR

Five non-pathological bone tissues were retrieved from surgical pathology files (two females and three males, 17 to 55 years old) of Department of Pathology, Tohoku University Hospital (Sendai, Japan).

Tissue sections were immunostained using a biotin-streptavidin method with Histofine kit (Nichirei Co. Ltd., Tokyo, Japan). The monoclonal antibody for AR (AR411) [23] was obtained from DakoCytomation (Kyoto, Japan). Experimental procedures employed in our present study have been previously described in detail [22,23]. The dilutions of primary AR antibody were 1:100. The antigen-antibody complex was then visualized with 3,3′-diaminobenzidine solution, and counterstained with hematoxylin. Prostate cancer was used as a positive control for AR. Normal mouse IgG was used as a negative control for immunostaining and no specific immunoreactivity was detected.

Statistical analysis

Results were expressed as mean±SD. Statistical analysis was performed with the StatView 5.0 J software (SAS Institute Inc., NC, USA). All data were analyzed by analysis of variance (ANOVA) followed by post hoc Bonferroni/Dunnett multiple comparison test. A p-value <0.05 was considered to indicate statistical significance.

Results

Characteristics of hFOB, MG-63, and Saos-2 cell line

Characteristics of osteoblast and osteoblast-like cell lines are summarized in Figs. 2A and B. hFOB cells expressed mRNA transcripts of AR and ERβ. Relatively low level of ERα mRNA transcript was detected in hFOB cells. Aromatase, 17β-HSD type 1, 3, and 5, and 5α-Red types 1 and 2 mRNA transcripts were all detected in hFOB cells by

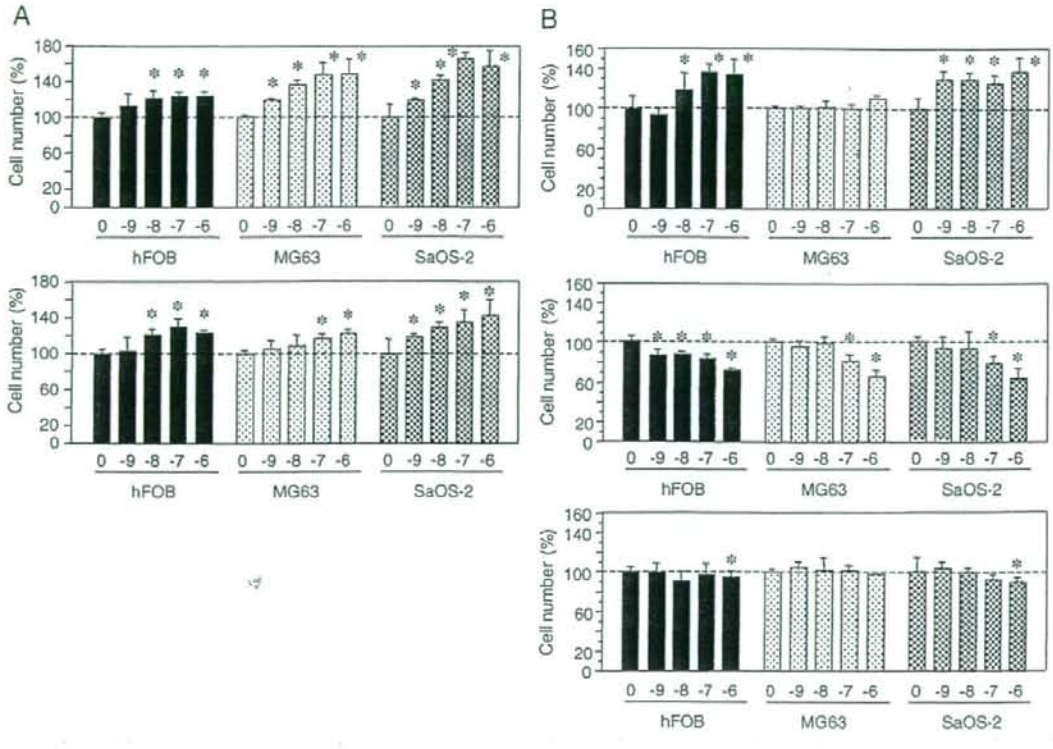

Fig. 3. (A) Proliferation of hFOB cells treated by estradiol (top) and 5α-DHT (bottom). *$p<0.05$ vs. vehicle control (0). (B) Proliferation of hFOB cells treated by exemestane (top), aminogluthetimide (middle), and Aromatase Inhibitor-I (bottom). *$p<0.05$ vs. vehicle control (0). $n=5$.

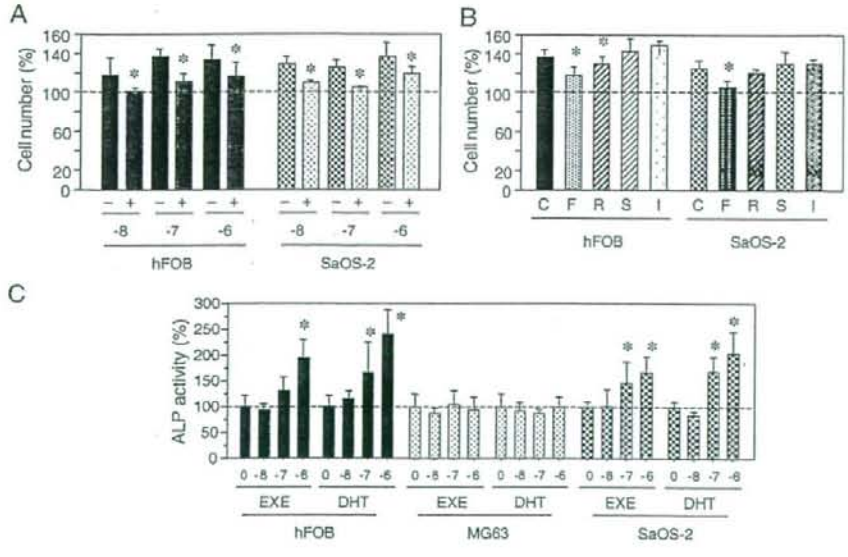

Fig. 4. (A) Effects of hydroxyflutamide on exemestane (10^{-8} to 10^{-6} M) stimulated the cell proliferation of both hFOB and Saos-2. With (+) or without (−) hydroxyflutamide, $p<0.05$ vs. without hydroxyflutamide (*). (B) Effects of steroid receptor blockers on exemestane (10^{-7} M) stimulated cell proliferation of hFOB and Saos-2. C, 10^{-7} M exemestane; F, hydroxyflutamide (5×10^{-6} M); R, RU38,486 (5×10^{-6} M); S, spironolactone (5×10^{-6} M); I, ICI182,720 (5×10^{-6} M). *$p<0.05$ vs. C (C) ALP activity in hFOB, Saos-2, MG-63 treated with exemestane (EXE, 10^{-8} to 10^{-6} M), or 5α-DHT (DHT, 10^{-8} to 10^{-6} M). *$p<0.05$ vs. vehicle control (0).

RT-PCR. Aromatase, ERα, ERβ, and AR were all detected in osteoblast-like cell lines, Saos-2 and MG-63 (Fig. 2B). In hFOB cell, expression of ERβ mRNA was more predominant than that of ERα mRNA. ERα mRNA as well as ERβ mRNA was detected in Saos-2 and MG-63 cells. The levels of AR mRNA expression in both hFOB and Saos-2 were significantly higher ($p=0.01$) than that in MG-63. ALP mRNA was also detected in intact hFOB, Saos-2, and MG-63 cells (data not present), respectively.

Estradiol and testosterone production

Results were summarized in Fig. 2C. The E2 levels in the medium of hFOB supplemented with Δ_4 treated with EXE or AI-I were significantly lower than that of cells without AIs. The levels of TST in the medium of hFOB supplemented with Δ4 treated with EXE or AI-I were significantly higher than that of cells without AIs.

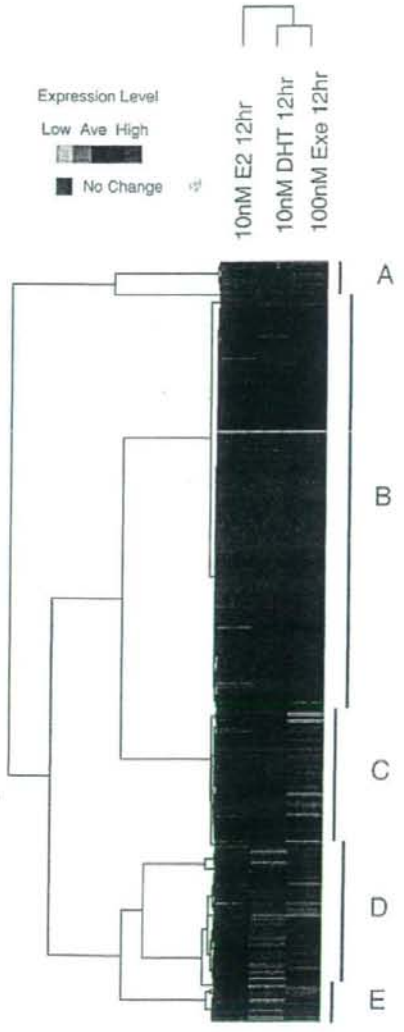

Fig. 5. In clustering analysis of the expression levels of each gene in hFOB cells treated with estradiol (E2), 5α-dihydrotestosterone (DHT), and exemestane (Exe).

Cell proliferation

Results of the cell proliferation assays are summarized in Figs. 3 and 4. There was a significant increment in the number of the cells after 72 h in hFOB, Saos-2, and MG-63 cells treated with 10^{-9} M (Saos-2 and MG-63) or 10^{-8} M (hFOB) to 10^{-6} M E2 (Fig. 3A). The cell number of hFOB and Saos-2 cells treated by 10^{-9} M (Saos-2) or 10^{-8} M (hFOB) to 10^{-6} DHT for 72 h was also significantly higher than control (Fig. 3A). The number of MG-63 cells was significantly increased only by high dose of DHT (10^{-7} M and 10^{-6} M) treatments (Fig. 3A). Prg (10^{-9} M to 10^{-6} M) treatments did not change the number of cells even after 72 h in all three cell lines examined (data not present).

Both EXE (Fig. 3B) and 17H-EXE (data not present) treatments of 10^{-8} M to 10^{-6} M, which were comparable to pharmacological inhibition doses of aromatization (Table 1), significantly increased the hFOB cell number for 72 h, respectively. In Saos-2 cells treated with relatively low dose, 10^{-9} to 10^{-6} M EXE, there was a significant increment in the number of the cells after 72 h (Fig. 3B). However, all the dose (10^{-9} M to 10^{-6} M) of EXE employed did not results in the change of cell number of MG-63 even after 72 h of treatment (Fig. 3B). The cell number of both hFOB and Saos-2 cells treated by both 10^{-6} M EXE and/or 17H-EXE for 48 h was also significantly higher than that treated for 24 h (data not present).

AGM treatment [10^{-9} (hFOB) or 10^{-7} (Saos-2 and MG-63) to 10^{-6} M] diminished the number of these three cells (Fig. 3B) and morphological changes in these cells were consistent with those caused by cytotoxic effects (data not present). AI-I treatment (10^{-9} to 10^{-7} M) was not associated with significant increment of the cell number in these cell lines (Fig. 3B). Only high dose (10^{-6} M) of AI-I significantly diminished the cell numbers of hFOB and Saos-2 but not of MG-63 (Fig. 3B).

The androgen receptor antagonist OHF (5×10^{-6} M) diminished the effects of EXE on these increments of both hFOB and Saos-2 cells (Figs. 4A and B). Treatment with RU but not spironolactone and ICI also inhibited EXE effects on hFOB cells (Fig. 4B).

ALP activity assay

Results of the ALP activity assay were summarized in Fig. 4C. There was a significant increment in the ALP activity of both hFOB and Saos-2 cells treated with 10^{-7} M (Saos-2) and/or 10^{-6} M (hFOB and Saos-2) EXE. Both 10^{-7} M and 10^{-6} M DHT treatment also increased the ALP activity in hFOB and Saos-2 cells, respectively. There were no changes of ALP activity in MG-63 treated with 10^{-8} M to 10^{-6} M of EXE and DHT, respectively.

Table 3a
Genes induced by exemestane treatment in hFOB cells—2.0 higher

	Gene title	Gene symbol	Raw data			Ratio	
			C	D	Ex	D	Ex
NM_002466	V-myb myeloblastosis viral oncogene homolog (avian)-like 2	**MYBL2**	70.9	156.9	150.3	2.2	2.1
AW444985	–	–	57.8	124.7	127.1	2.2	2.2
AF143684	Myosin IXB	MYO9B	48.3	64.4	122.2	1.3	2.5
NM_024682	TBC1 domain family, member 17	TBC1D17	31.7	37.6	64.8	1.2	2.0
BE965311	Chromosome 16 open reading frame 23	C16orf23	29.2	44.2	64.0	1.5	2.2
NM_004233	CD83 antigen (activated B lymphocytes, immunoglobulin superfamily)	CD83	29.0	66.5	60.9	2.3	2.1
AI806031	Skeletal muscle and kidney enriched inositol phosphatase	SKIP	27.7	48.6	55.4	1.8	2.0
AL136729	Ring finger protein 123	RNF123	20.0	23.7	41.3	1.2	2.1
NM_015254	Kinesin family member 13B	KIF13B	13.0	24.5	39.4	1.9	3.0
AL110249	Chromosome 20 open reading frame 194	C20orf194	13.4	39.0	29.7	2.9	2.2
AF208502	Early B-cell factor	EBF	12.5	21.1	28.5	1.7	2.3
AW007221	Solute carrier family 13 (sodium/sulfate symporters), member 4	SLC13A4	12.3	9.6	27.8	0.8	2.3
AB007458	TP53 activated protein 1	TP53AP1	12.6	22.2	26.2	1.8	2.1
AV713913	Osteopetrosis associated transmembrane protein 1	**OSTM1**	9.8	16.5	21.3	1.7	2.2
BF339201	THAP domain containing 6	THAP6	6.0	14.0	20.6	2.3	3.4
AK000455	Hypothetical gene MGC16733 similar to CG12113	MGC16733	7.3	16.6	18.8	2.3	2.6
AW974816	–	–	2.2	16.0	17.2	7.2	7.7
AK025325	Transcribed locus, moderately similar to NP_689573.2 zinc finger protein 573	–	7.3	11.4	16.2	1.6	2.2
NM_021192	Homeo box D11	**HOXD11**	5.3	16.2	15.8	3.0	3.0
NM_022169	ATP-binding cassette, sub-family G (WHITE), member 4	ABCG4	7.0	10.5	15.7	1.5	2.2
R62907	Disabled homolog 2, mitogen-responsive phosphoprotein (*Drosophila*)	DAB2	7.7	13.0	15.5	1.7	2.0
NM_002661	Phospholipase C, gamma 2 (phosphatidylinositol-specific)	PLCG2	7.3	12.3	15.3	1.7	2.1
BG393032	Solute carrier family 13 (sodium/sulfate symporters), member 4	SLC13A4	6.4	6.7	15.1	1.0	2.3
BC002794	Tumor necrosis factor receptor superfamily, member 14	TNFRSF14	6.2	11.3	13.6	1.8	2.2
BC042908	KIAA0690	KIAA0690	5.6	7.4	13.5	1.3	2.4
AW451961	Adenylate cyclase activating polypeptide 1 (pituitary) receptor type I	**ADCYAP1R1**	4.3	11.7	13.2	2.7	3.1
AI863264	Glypican 2 (cerebroglycan)	**GPC2**	5.3	7.2	13.2	1.3	2.5
AF130050	ACA47 scaRNA gene	-	5.6	9.5	12.9	1.7	2.3
AK022326	Hypothetical gene supported by AK022326	-	6.1	12.7	12.9	2.1	2.1
AK021807	Low density lipoprotein receptor-related protein 11	LRP11	5.9	6.2	12.8	1.0	2.2
AU155415	Kallikrein 7 (chymotryptic, stratum corneum)	KLK7	5.6	13.5	12.7	2.4	2.3
BF673779	Hypothetical protein FLJ30834	FLJ30834	5.5	6.3	12.3	1.1	2.2
AV646335	–	–	2.6	13.0	11.2	5.0	4.3
BC040600	–	–	5.0	5.4	10.6	1.1	2.1
AI131035	–	–	5.1	9.2	10.5	1.8	2.1

C, vehicle control; D, 5α-dihydrotestosterone; Ex, exemestane. Genes that performed quantitative RT-PCR were described in bold style.

Microarray/clustering analysis

In hFOB cells, the hierarchical clustering analysis contains 430 genes which demonstrated expression ratios above 2.0-fold and below 0.5-fold compared with vehicle control cells after 12 h of each gene treated with 10^{-8} M E2, 10^{-8} M DHT, or 10^{-7} M EXE. The expression profiles of EXE treated cells were closely related to those of DHT (Fig. 5). In this study, we focused on 35 genes (Table 3a), which were all up-regulated twice or more than control. In this group, we further focused on 5 genes, B-Myb 2 (MYBL2), osteopetrosis associated transmembrane protein 1 (OSTM1), homeo box D 11 (HOXD11), adenylate cyclase activating polypeptide 1 receptor (ADCYAP1R1), and glypican 2 (GPC2) which are all considered to play important roles in EXE or DHT induced cell proliferation. We therefore examined whether these 5 genes were increased by EXE or DHT treatments using quantitative RT-PCR in hFOB cells. We also examined the validation of results of microarray analysis obtained in hFOB cells in Saos-2 and MG-63 cells.

Validation of microarray analysis using quantitative RT-PCR

In hFOB cells, all of these 5 genes described above were significantly increased by 10^{-7} M EXE treatment, and 3/5 genes (except for OSTM1 and GPC2) were also significantly increased by 10^{-8} M DHT treatment. HOXD11 and ADCYAP1R1 genes increased by both EXE and DHT were significantly diminished by OHF (5×10^{-6} M) treatment (Figs. 6A–C).

The similar results of changes of MYBL2 expression were also obtained in both Saos-2 and MG-63 treated with EXE and DHT, respectively (Fig. 6A). In addition, the results of HOXD11 expression in hFOB were equivalent to those in Saos-2 but not in MG-63 treated with EXE and DHT (Fig. 6B). Other genes induced by treatment of EXE and DHT in hFOB such as OSTM1, GPC2, and ADCYAP1R1 were not changed in both Saos-2 and MG-63 cells treated with EXE and DHT, respectively (data not present). AI-I or AGM treatment did not increase all of these genes expression in hFOB (data not present).

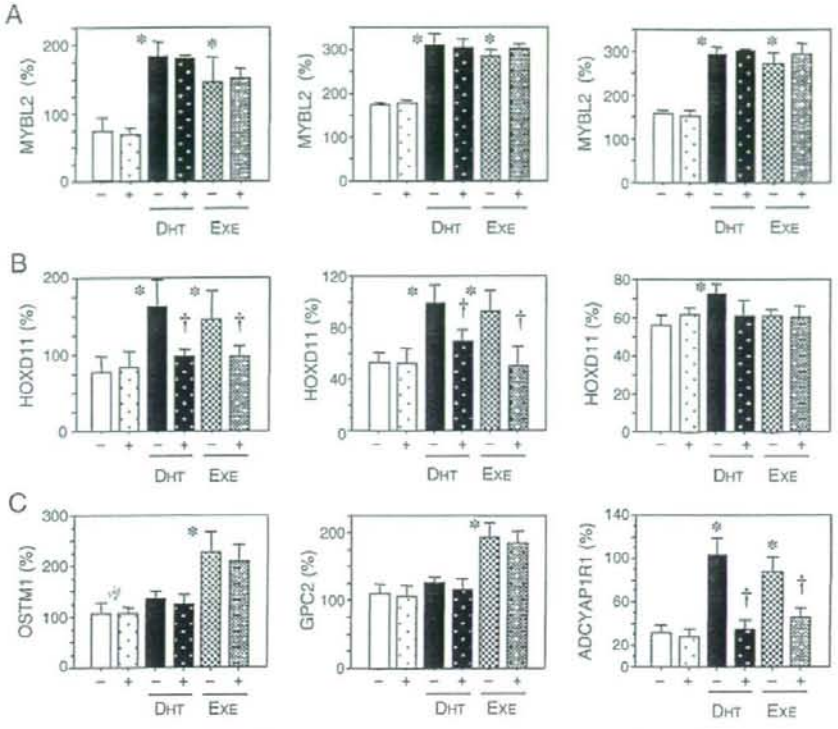

Fig. 6. Validation of microarray analysis. (A) Expression levels of MYBL2 in hFOB (left), Saos-2 (middle), and MG-63 (right). (B) Expression levels of HOXD11 in hFOB (left), Saos-2 (middle), and MG-63 (right). (C) Expression levels of OSTM1, GPC2, and ADCYAP1R1 in hFOB. DHT: 10^{-8} M 5α-dihydrotestosterone, EXE: 10^{-7} M Exemestane, with (+) or without (−) hydroxyflutamide, $p<0.05$ vs. control (*) or without hydroxyflutamide (†).

Analysis of osteoblast growth-related genes

Results of microarray analysis in hFOB cell demonstrated that osteoblast growth-related genes [24,25] such as COL1A1, SMAD1, SMAD5, SPARC, and RUNX2 were all up-regulated by exemestane (10^{-7} M) treatment but the degrees of increment were all under 2-fold (Table 3b). In this microarray analysis, other expression levels of previously reported osteoblast-related genes were not altered.

In hFOB cells, the validation analysis of these genes described above using quantitative RT-PCR (Fig. 7) demonstrated that 4/5 genes (except for COL1A1) were significantly increased by 10^{-7} M EXE treatment, and 4/5 genes (except for SMAD1) were also significantly increased by 10^{-8} M DHT treatment. The increased expression of the SMAD1, SMAD5, and SPARC genes by EXE or DHT, was significantly diminished by OHF (5×10^{-6} M) treatment. There were no effects of OHF pretreatment on the increased expression levels of RUNX2 that had occurred after both EXE and DHT treatments. Both AI-I and AGM treatment could not increase all of these genes expression in hFOB (data not present).

In Saos-2 cells, 4/5 genes (except for RUNX2) were significantly increased by 10^{-7} M EXE treatment, and 3/5 genes (except for RUNX2 and SMAD1) were also significantly increased by 10^{-8} M DHT treatment. The increment of the COL1A1, SMAD5, and SPARC genes expression by EXE or DHT, was significantly diminished by OHF (5×10^{-6} M) treatment. All of these 5 genes did not change in MG-63 cells treated with EXE or DHT, respectively (data not present).

Immunohistochemistry of AR

Marked AR immunoreactivity was detected in the nuclei of osteoblasts or lining cells but not in osteoclasts in four cases (Fig. 8). In these four cases, AR immunoreactivity was also detected in osteocytes and condrocytes. In one case, there was no immunoreactivity in all types of bone cells.

Discussion

In the clinical study of EXE compared to placebo administrated for two years [26,27], EXE modestly enhanced bone loss from the femoral neck without significant influence on lumber bone loss despite a marked systemic estrogen depletion. Furthermore, the risks of clinical bone fractures are considered to be lower with EXE treatment than that seen with non steroidal AIs [27,28], though it is also important to recognize that EXE has not been shown to significantly increase the amount of bone mass in various clinical studies of breast cancer patients [26,27]. The relative protective effect of EXE, a

Table 3b
Genes induced by exemestane treatment in hFOB cells—the osteoblast growth-related genes

	Gene title	Gene symbol	Raw data			Ratio	
			C	D	Ex	D	Ex
K01228	Collagen, type I, alpha 1	**COL1A1**	2797.2	3240.9	3058.5	1.2	1.1
BE221212	Collagen, type I, alpha 1	**COL1A1**	2741.1	3048.3	3052.2	1.1	1.1
AI743621	Collagen, type I, alpha 1	**COL1A1**	228.0	241.6	242.5	1.1	1.1
AA788711	Collagen, type I, alpha 2	COL1A2	2250.6	2474.3	2375.4	1.1	1.1
NM_000089	Collagen, type I, alpha 2	COL1A2	1749.1	1848.7	1787.6	1.1	1.0
M60485	Fibroblast growth factor receptor 1	FGFR1	178.9	185.7	198.6	1.0	1.1
BE467261	Fibroblast growth factor receptor 1	FGFR1	165.4	208.6	189.7	1.3	1.1
M63889	Fibroblast growth factor receptor 1	FGFR1	119.3	111.6	140.5	0.9	1.2
NM_023110	Fibroblast growth factor receptor 1	FGFR1	60.5	84.0	70.2	1.4	1.2
AU145411	Fibroblast growth factor receptor 1	FGFR1	29.2	44.1	37.5	1.5	1.3
AI359368	Fibroblast growth factor receptor 3	FGFR3	41.4	65.5	58.7	1.6	1.4
NM_001552	Insulin-like growth factor binding protein 4	IGFBP4	809.1	1027.5	1040.4	1.3	1.3
AL353944	Runt-related transcription factor 2	**RUNX2**	192.9	226.3	216.3	1.2	1.1
AU146891	SMAD, mothers against DPP homolog 1 (*Drosophila*)	**SMAD1**	161.2	195.6	204.6	1.2	1.3
NM_005901	SMAD, mothers against DPP homolog 2 (*Drosophila*)	SMAD2	100.3	108.2	113.7	1.1	1.1
NM_005902	SMAD, mothers against DPP homolog 3 (*Drosophila*)	SMAD3	110.2	106.5	127.7	1.0	1.2
BF526175	SMAD, mothers against DPP homolog 5 (*Drosophila*)	**SMAD5**	361.0	488.3	514.2	1.4	1.4
AI478523	SMAD, mothers against DPP homolog 5 (*Drosophila*)	SMAD5	300.7	384.9	346.9	1.3	1.2
AF010601	SMAD, mothers against DPP homolog 5 (*Drosophila*)	SMAD5	79.2	99.7	87.6	1.3	1.1
AY014180	SMAD-specific E3 ubiquitin protein ligase 2	SMURF2	804.2	844.7	851.8	1.1	1.1
AU157259	SMAD-specific E3 ubjquitin protein ligase 2	SMURF2	77.1	81.5	86.3	1.1	1.1
AL575922	Secreted protein, acidic, cysteine-rich (osteonectin)	**SPARC**	1702.1	1935.5	1925.8	1.1	1.1
BF508662	Sprouty homolog 1, antagonist of FGF signaling (*Drosophila*)	SPRY1	31.9	46.3	45.4	1.5	1.4
NM_014886	TGF beta-inducible nuclear protein 1	TINP1	1185.7	1259.5	1241.1	1.1	1.0

C, vehicle control; D, 5α-dihydrotestosterone; Ex, exemestane. Genes that performed quantitative RT-PCR were described in bold style.

steroidal aromatase inhibitor, has been therefore attributed to its actions through AR in osteoblasts. Systemic androgenic effects such as hypertricosis, hair loss, hoarseness, and acne have been reported only in 4% [6] of the patients treated with EXE (25 mg/day) and the frequency of these effects increases to approximately 10% in those treated with higher dose 200 mg/day of EXE [6]. This finding suggests that the patients treated with EXE are under relatively weak systemic androgenic effects. Androgen sensitivity has been well-known to be subject to great individual variation caused by AR gene CAG polymorphism in women as well as men [29,30]. Therefore, this 5 to 10% of the patients who manifested clinical androgenic effects by EXE treatment may be individuals associated with relatively enhanced androgenic sensitivity. Replacement therapy with TST is generally effective at restoring bone in hypogonadal men [31]. In female-to-male, genetic female transsexual subjects, high-dose TST therapy generally increased BMD at the femoral neck, despite decrement of E2 to postmenopausal levels [32,33]. Therefore, androgens may play an important role in bone protection in women as well as men.

The results of cell proliferation assay demonstrated that the cell number of MG-63 was increased by both E2 and DHT treatments, but the dose of DHT was relatively higher than that in two other cells. MG-63 expressed higher levels of ERα/β mRNA, but the level of AR mRNA was lower than that in both Saos-2 and hFOB. Both cell proliferation and ALP activity of MG-63 could not be stimulated by EXE treatment. Molecular mechanisms of androgen actions on osteoblasts have remained largely unknown. Androgen is well-known to stimulate osteoblast proliferation [34] and differentiation [35]. For instance, osteoprotegerin mRNA was increased by TST as well as DHT treatments in mouse 3T3-E1 cells [36].

AR and ERβ but not ERα are predominantly detected in osteoblasts located on human cancellous bone using immunohistochemical analysis [37]. Therefore, hFOB examined in this study is considered to maintain relatively native status of sex steroids pathways in human osteoblasts. Therefore, we employed hFOB for further examination of EXE effects on osteoblast gene expression pattern using microarray analysis. In this study, we demonstrated that the genes MYBL2 [38], OSTM1 [39], HOXD11 [40], ADCYAP1R1 [41], and GPC2 [42] were target genes of EXE alone or both EXE and DHT in hFOB using microarray/PCR analysis. These genes were demonstrated to be involved in regulation of cell cycle, differentiation, and transcription. In EXE or DHT treatment in hFOB and Saos-2, in which cells proliferations were stimulated, an increased expression of HOXD11 gene was detected. The product of the mouse Hoxd11 gene was reported to play a role in forelimb morphogenesis [40,43]. Therefore, these finding suggest that osteoblast cell proliferation stimulated by EXE treatment may depend on HOXD11 gene expression through AR. In this study, the cell proliferation of MG-63, which expressed relatively low level of AR, was not stimulated by EXE. In addition, HOXD11 gene expression was not up-regulated by EXE treatment in MG-63 cells. These results were also consist with the protective effects of EXE through potential androgen-HOXD11 pathway in osteoblast cells. In this study, we also examined the effects of EXE and DHT on osteoblast growth-related genes using micro-

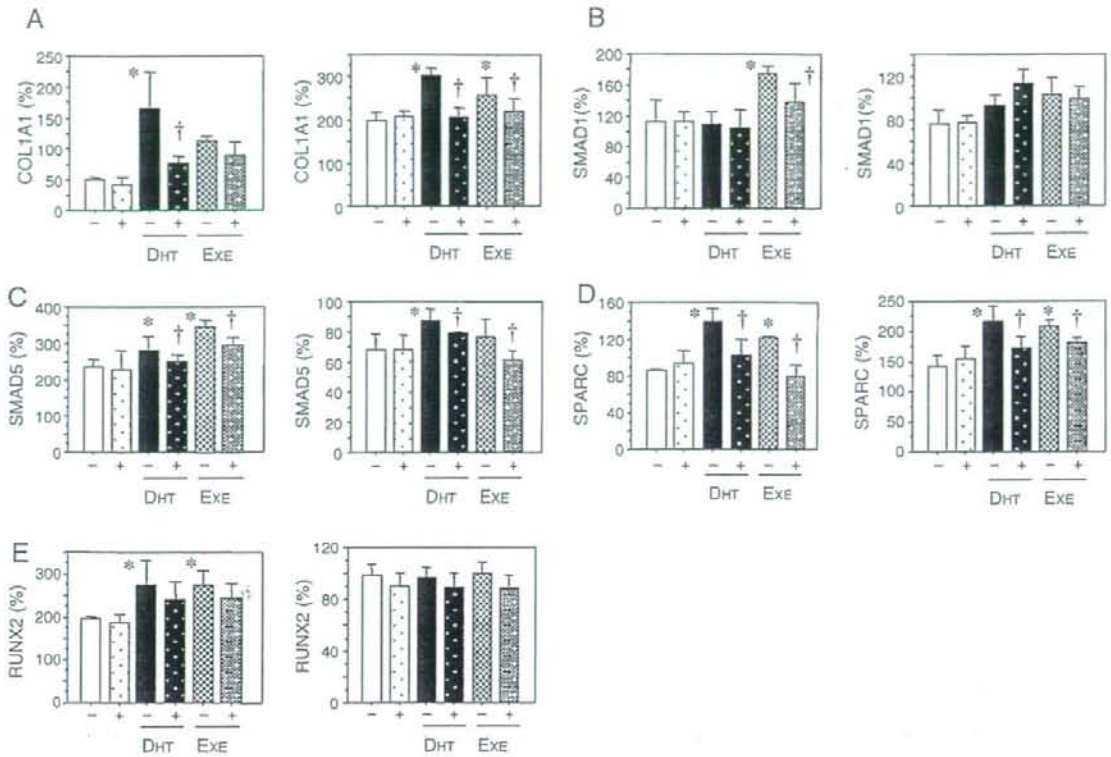

Fig. 7. Expression levels of osteoblast growth-related genes in hFOB (left) and Saos-2 (right). DHT: 10^{-8} M 5α-dihydrotestosterone, EXE: 10^{-7} M Exemestane, with (+) or without (−) hydroxyflutamide, $p<0.05$ vs. control (*) or without hydroxyflutamide (†).

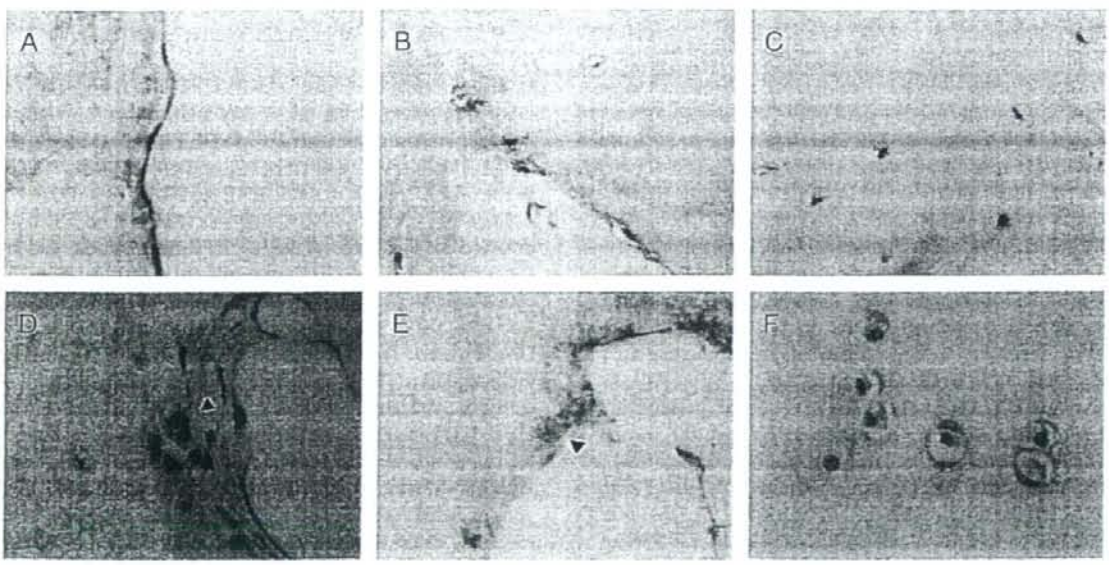

Fig. 8. Immunohistochemistry of androgen receptor in human bone tissues. Immunoreactivity of androgen receptor was detected in nuclei of osteoblasts/liner cells (A, B) but not in osteoclasts (D, E; arrowheads). Immunoreactivity of androgen receptor was also detected in nuclei of osteocytes (C) and condrocytes (F).

array analysis and following quantitative RT-PCR. COL1A1, SMAD5, and SPARC (osteonectine) were up-regulated by EXE and/or DHT treatments in both hFOB and Saos-2 cells. EXE or DHT treatments in both hFOB and Saos-2 also resulted in increased ALP activity. There have been, however, no studies reported on whether these genes are primary or secondary androgen responsive genes in osteoblasts. The AR-specific antagonist, OHF demonstrated no inhibitory effects on RUNX2 expression increased by EXE or DHT treatment in hFOB cells. In addition, hFOB cell growth induced by high dose of EXE treatment was not completely inhibited by OHF treatment. These results all suggest that EXE also may stimulate hFOB cell proliferation through both AR dependent and independent pathways. From our data of steroid production in hFOB, EXE may have an additional androgenic effect through increased TST levels in conjunction with inhibition of aromatization in hFOB cells. However, it awaits further investigations for clarification.

In normal bone remodeling, bone formation by osteoblasts follows bone resorption by osteoclasts and occurs in a precise and quantitative manner (coupling). In this coupling between bone formation and resorption, a coupling factor that induces bone formation is considered to be released during osteoclastic bone resorption [44]. This study has focused on the specific effects on osteoblast cells. However, it is true that there were significant increases in both serum bone formation and resorption markers in postmenopausal women administered with EXE for 2 years [26]. Osteoclasts, which are responsible for bone resorption, are target cells for many anti-osteoporosis therapeutic agents such as bisphosphonate of postmenopausal women [45]. However, it is unclear whether EXE acts on osteoclast directly. Chen et al. [46] reported that testosterone inhibited osteoclast formation stimulated by parathyroid hormone through the AR but not through the production of intrinsic estrogen using primary mouse osteoclast cells. In both human and rodent bone tissues, AR is expressed in both osteoblasts and osteocytes [47,48]. However, AR is detected in osteoclasts of rodent but not in human cells [31,47,48]. Therefore, in humans, androgens are considered to exert their effects on bone through osteoblasts. EXE may therefore exert its possible androgenic effects on human bone through osteoblasts but not osteoclasts. Results of our present study also suggest the possible roles of EXE on osteoblast cells through AR independent manner. Results of clinical studies suggest that the combination therapy of AI and COX-2 inhibitors could provide more effective aromatase inhibition than single therapy in hormone-sensitive postmenopausal breast cancer [49]. Bone resorption induced by IL-1 and IL-6 was also reported to occur via stimulation of COX-2 dependent PGE_2 production in osteoblasts *in vitro* [50]. Therefore, further investigations are required to clarify the effects of AI including EXE on human bone tissues.

In summary, this study using osteoblast and osteoblast-like cell lines suggested the potential protective effect of steroidal AI, EXE on osteoblasts occurred through both AR dependent and independent pathways. HOXD11 gene known as bone morphogenesis factor and osteoblast growth-related genes were induced by EXE treatment as well as DHT treatment in both hFOB and Saos-2. Damages of bone tissues by estrogen depletion caused by AI administration are considered unavoidable but the selection of potential hormone therapies which could minimize the damages or injuries of bone tissues is considered important.

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Prognoses and Prognostic Factors of Carcinosarcoma, Endometrial Stromal Sarcoma and Uterine Leiomyosarcoma: A Comparison with Uterine Endometrial Adenocarcinoma

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Key Words
Uterine sarcoma · Prognostic factors · Endometrial cancer · Carcinosarcoma · Monoclonal

Abstract
Aims: The aims of this study were to evaluate the factors affecting prognosis in patients with uterine sarcomas and to demonstrate that carcinosarcoma bears a similarity to high-grade endometrial carcinoma in terms of its prognosis and clinicopathological parameters. ***Methods:*** In June 2004, 17 Japanese institutions received questionnaires regarding uterine sarcomas. Study patients had uterine sarcomas initially treated at each institution between January 1990 and May 2004. Survival analyses and comparisons were performed by univariate methods. Patient data of 921 cases of endometrial adenocarcinoma were also analyzed and compared to the data with the uterine sarcomas. ***Results:*** One hundred twenty-one patients with uterine sarcomas were identified who met study eligibility criteria. In uterine sarcomas, carcinosarcoma had a worse prognosis than other sarcomas, but the difference was not significant ($p = 0.302$). In carcinosarcoma, significant differences were observed with age ($p = 0.0388$), stage ($p < 0.01$) and surgical procedure (with or without pelvic lymphadenectomy, $p = 0.0316$). In carcinosarcoma and G3 adenocarcinoma, no significant difference was identified with regard to overall survival in univariate ($p = 0.191$) and multivariate ($p = 0.168$) analyses. ***Conclusion:*** Our results demonstrate that the clinical behavior of carcinosarcoma strongly resembles that of G3 endometrial adenocarcinoma, setting it apart from other 'pure' uterine sarcomas.

Introduction

Uterine sarcomas are rare, accounting for only 3–5% of all malignant uterine neoplasms [1, 2]. Traditionally, uterine sarcomas have been classified into 3 main histologic subgroups, in order of decreasing incidence: carcinosarcoma (CS), leiomyosarcoma (LMS) and endometri-

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al stromal sarcoma (ESS). Each group of tumors is distinct with respect to pattern of spread, pathologic features, prognostic factors and response to treatment [3].

Various factors affect the prognosis of uterine sarcomas, the most important being the extent of spread or stage at diagnosis [4–8]. The contribution of other factors, including age at diagnosis, menopausal status and mitotic count, remains controversial [5, 9, 10]. An improved understanding of what affects CS prognosis would improve survival by facilitating the development of more effective, targeted therapies.

CS, also referred to as a malignant mixed mullerian tumor [11], is a subtype of uterine sarcoma. The adjuvant treatment for this neoplasm is similar to that used for high-grade uterine sarcomas such as LMS. There are 4 main theories regarding the histogenesis of CS: (1) the collision theory, (2) the combination theory, (3) the conversion theory and (4) the composition theory [12]. Several argue that CS arises through conversion and metaplasia [13–15], and should be grouped with epithelial tumors based on histopathological data.

The aims of this study were to evaluate the factors affecting prognosis in patients with uterine sarcomas and to demonstrate that CS bears a similarity to high-grade endometrial carcinoma in terms of its prognosis and clinicopathological parameters.

Materials and Methods

In June 2004, 17 Japanese institutions (Fukushima Medical University Hospital, Gonohe Municipal Hospital, Hachinohe City Hospital, Hanamaki-Kousei Hospital, Ichinoseki Hospital, Kesennuma City Hospital, Miyagi Cancer Center, NTT-East Hospital, Ohta-Nishinouchi Hospital, Sendai Medical Center, Takeda General Hospital, Tohoku Kosai Hospital, Tohoku Kousei-Nenkin Hospital, Tohoku University Hospital, Tome City Sanuma Hospital, Yamagata Prefectural Central Hospital and Yuri General Hospital) received questionnaires regarding uterine sarcomas (CS, LMS and ESS). All participating institutions met the requirements of the Ministry of Health and Welfare of Japan and were considered to be specialized centers for gynecologic oncology. Patient data were collected through chart reviews conducted by a study representative from each institution.

Study patients were those with uterine sarcomas, initially treated at each institution between January 1990 and May 2004. Data including age, tumor histologic subtype, stage, treatments (surgical procedure, chemotherapy and radiation), recurrence and outcome were collected. Histologic subtype was determined according to the WHO criteria. Patient data of 921 cases of endometrial adenocarcinoma (all cases were of the endometrioid subtype: G1, 581 cases; G2, 240 cases; G3, 100 cases), initially treated at each institution within the same period, were analyzed and compared with the uterine sarcoma data.

Survival curves were estimated using the Kaplan-Meier method [16]. Overall survival was calculated from the time of initial admission for the treatment of disease. Survival times of patients still alive or lost to follow-up were censored in May 2004. Survival differences and associations of histological subtypes and other patient characteristics were analyzed by the log-rank test [17]. The Cox proportional hazards model was used to identify independent prognostic factors, with adjustments for various prognostic factors [18].

Results

One hundred twenty-one patients with uterine sarcomas (CS, 71 cases; LMS, 31 cases; ESS, 19 cases) who met study eligibility criteria were identified. The uterine sarcoma and endometrial adenocarcinoma patient characteristics are summarized in table 1. Patients with CS were older than patients with LMS and ESS, and similar in age to patients with G3 in endometrial adenocarcinoma. In CS, more than half the patients received surgery with pelvic and/or para-aortic lymphadenectomy, while most patients received surgery without lymphadenectomy in LMS and ESS. In ESS, most patients (78.9%) were diagnosed at an early stage (stage I/II) compared with CS (49.3%) and LMS (58.0%, $p < 0.05$). In patients who received chemotherapy, the chemotherapy was typically platinum based, in combination with ifosfamide (mainly in sarcoma patients) and adriamycin (mainly in endometrial adenocarcinoma patients). Recently, a relatively small number of patients received combination of carboplatin and paclitaxel.

Patient follow-up ranged from 4 to 110 months (median 47.5 months). Figure 1 shows the Kaplan-Meier survival analysis for all endometrial adenocarcinomas subdivided by grade (fig. 1). Median survival time was not calculated, but the difference in overall survival between G3 and G1/2 was significant ($p < 0.01$). Figure 2 shows the survival curves for all uterine sarcomas subdivided by histological subtype (CS, LMS and ESS). CS tended to have a worse prognosis in comparison to LMS and ESS, but the difference was not significant ($p = 0.302$). Table 2 shows the results of the univariate analysis for overall survival in patients with CS. Significant differences were observed with regard to age ($p < 0.05$), stage ($p < 0.01$) and surgical procedure (with or without pelvic lymphadenectomy, $p < 0.05$; fig. 3). Tables 3 and 4 show the results of the univariate analysis for overall survival in patients with LMS and ESS, respectively. Of the variables analyzed, only stage (I/II versus III/IV) significantly predicted overall survival ($p < 0.0001$ for LMS and $p < 0.05$ for ESS; fig. 4, 5).

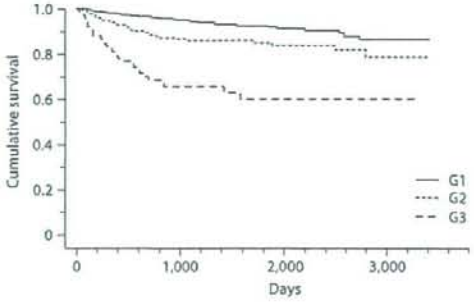

Fig. 1. Overall survival for endometrial cancer shown by Kaplan-Meier survival curves for all endometrial adenocarcinoma subdivided by grade.

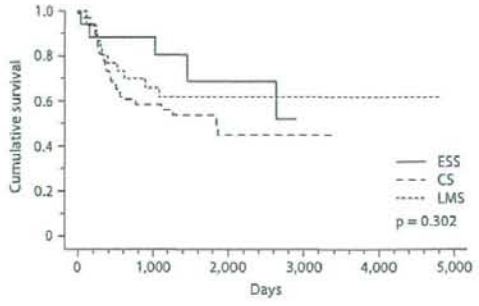

Fig. 2. Overall survival for uterine sarcomas shown by Kaplan-Meier survival curves for all uterine sarcomas subdivided by histological subtype.

Table 1. Patient characteristics of uterine sarcomas and endometrial adenocarcinoma

	LMS	ESS	CS	G1	G2	G3
Patients	31	19	71	581	240	100
Age, years	51.6 ± 9.9	49.6 ± 14.3	62.4 ± 11.3	54.7 ± 10.3	56.0 ± 10.2	57.7 ± 10.5
Range, years	27–73	30–80	28–89	24–86	27–81	31–88
<50 years	14	12	8	179	56	17
≥50 years	17	7	63	402	184	83
Stage						
I	17	14	28	454	155	40
II	1	1	7	47	20	15
III	8	2	24	71	54	32
IV	5	2	12	9	11	13
Operation						
TAH+BSO+LA	3	4	43	541	198	73
TAH+BSO	27	11	23	28	32	15
None	1	4	5	12	10	12
Chemotherapy						
Yes	21	11	46	125	90	58
No	10	8	25	456	150	42
Radiation						
Yes	7	2	8	63	41	27
No	24	17	63	518	199	73

TAH = Total abdominal hysterectomy; BSO = bilateral salpingo-oophorectomy; LA = lymphadenectomy.

The Kaplan-Meier curves for CS and G3 endometrial adenocarcinoma are compared in figure 6, and the results of multivariate analysis are shown in table 5. The 2 histological subtypes were similar with regard to overall survival in the univariate analysis (p = 0.191). In a multivariate analysis that included age, stage and surgical procedures with lymphadenectomy, the hazard ratio of overall survival for CS was 1.029 against that for G3 and this was not statistically significant (p = 0.168). The similarities not only in patient characteristics, but also in survival suggest that these 2 histological subtypes share a common etiology.

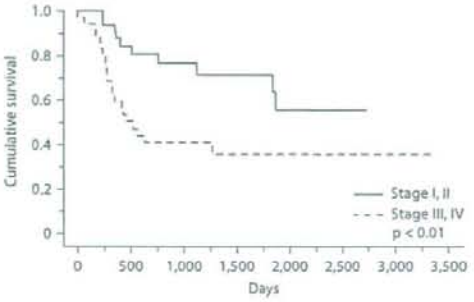

Fig. 3. Overall survival for patients with CS presented by Kaplan-Meier survival curves for CS subdivided by tumor stage.

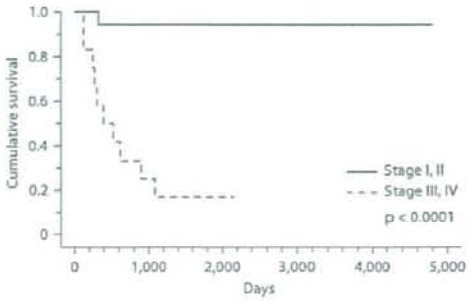

Fig. 4. Overall survival for patients with LMS shown by Kaplan-Meier survival curves for LMS subdivided by tumor stage.

Table 2. Univariate analysis of overall survival in patients with CS

	Hazard ratio	95% CI	p value
Age (<50 vs. ≥50 years)	1.038	1.002–1.075	0.0388
Stage (I/II vs. III/IV)	0.349	0.163–0.746	<0.01
Hysterectomy	0.592	0.178–1.972	0.3933
PLA	0.458	0.224–0.933	0.0316
PALA	0.212	0.029–1.565	0.1284

CI = Confidence interval; PLA = pelvic lymphadenectomy; PALA = para-aortic lymphadenectomy.

Table 4. Univariate analysis of overall survival in patients with ESS

	Hazard ratio	95% CI	p value
Age (<50 vs. ≥50 years)	0.995	0.913–1.084	0.9012
Stage (I/II vs. III/IV)	0.093	0.008–1.095	<0.05
Hysterectomy	0.612	0.063–5.959	0.6721
PLA	4.014	0.554–29.092	0.169
Chemotherapy	0.118	0.012–1.205	0.0714
Radiation	7.211	0.449–115.750	0.163

CI = Confidence interval; PLA = pelvic lymphadenectomy.

Table 3. Univariate analysis of overall survival in patients with LMS

	Hazard ratio	95% CI	p value
Age (<50 vs. ≥50 years)	0.993	0.933–1.056	0.813
Stage (I/II vs. III/IV)	0.04	0.005–0.317	<0.0001
PLA	0.84	0.107–6.572	0.868
PALA	5.282	0.617–45.245	0.128
Chemotherapy	1.008	0.294–3.458	0.989

CI = Confidence interval; PLA = pelvic lymphadenectomy; PALA = para-aortic lymphadenectomy.

Table 5. Multivariate analysis of overall survival in patients with uterine tumor

	Hazard ratio	95% CI	p value
G3 adenocarcinoma	1		
CS	1.029	0.988–1.071	0.168
Age (<50 vs. ≥50 years)	1.211	0.933–1.571	0.15
Stage (I/II vs. III/IV)	1.229	0.442–3.419	0.6929
Hysterectomy	0.5	0.244–1.021	0.057
Lymphadenectomy	0.428	0.237–0.772	0.0048

CI = Confidence interval.

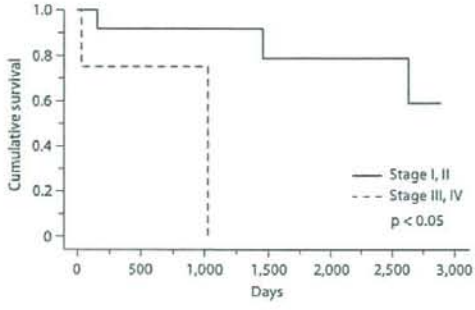

Fig. 5. Overall survival for patients with ESS presented by Kaplan-Meier survival curves for ESS subdivided by tumor stage.

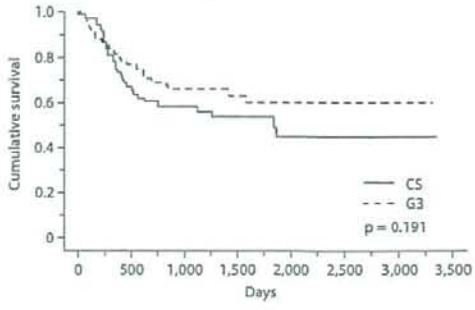

Fig. 6. Overall survival for patients with G3 endometrial adenocarcinoma and CS presented by Kaplan-Meier survival curves comparing G3 endometrial adenocarcinoma and CS.

Discussion

CS historically has been considered a subtype of uterine sarcoma, and for this reason it has been managed in a manner similar to high-grade uterine sarcomas such as LMS. The presence of an epithelial component renders this tumor fundamentally different from other uterine sarcomas, and it is this carcinomatous component that is the defining feature of CS [12]. The results of both univariate and multivariate analyses in this study demonstrate that CS is also clinically different from LMS and ESS with regard to patient age, stage at presentation, surgical treatment and prognosis. The clinical parameters of CS more closely resemble those of G3 endometrial adenocarcinoma.

In our study, the 5-year overall survival rates of ESS, LMS and CS were 68.4, 61.3 and 52.1%, respectively. In previous studies, the 5-year survival rates of ESS, LMS and CS were 61–69% [9, 19, 20], 62–73% [4, 6, 20] and 43–78.8% [20, 21], respectively. All these studies, as well as our study, identified tumor stage as a strong prognostic factor. The complete absence of residual disease following surgery, however, may be an even more important prognostic factor than stage [20]. Accordingly, if the cancer is diagnosed early and is still localized, therapy may be quite successful and can result in a high 5-year overall survival. In our study, a key difference between CS and ESS/LMS was that pelvic lymphadenectomy affected the prognosis in CS. Pelvic lymphadenectomy also improves survival in endometrial cancer [22–24]. The results of our study suggest that CS, like endometrial cancer, may spread via the lymphatics and this may reflect the dominance of its carcinomatous component.

A combination of conversion and metaplasia has been proposed to explain the histological origin of CS. The carcinomatous component is thought to be the true malignancy, and the sarcomatous component a consequence of either carcinomatous component-divergent differentiation [25] or metaplastic tumor change [13–15]. Recent epidemiological studies demonstrate that CS and endometrial adenocarcinoma share a similar risk factor profile, with both neoplasms being associated with obesity, exogenous estrogen use and nulliparity. Oral contraceptive use protects against the development of both tumor types [26–28]. The pattern of metastasis of CS more closely resembles that of a high-grade endometrial adenocarcinoma than that of 'pure' uterine sarcomas such as LMS. CS, like endometrial adenocarcinoma, primarily spreads via the lymphatics, whereas pure sarcomas commonly metastasize hematogeneously [14, 29, 30]. Immunohistochemical studies demonstrate that both the sarcomatous and carcinomatous components in CS coexpress cytokeratins and vimentin [31–36]. Additionally, both the carcinomatous and sarcomatous components in CS stain for p53 [37, 38]. These observations support a common origin for the epithelial and mesenchymal components because, if these were true collision tumors, such concordance in all cases would be extremely unlikely. Indeed, the pattern of X chromosome activation present in microdissected epithelial and stromal components [39, 40], and the pattern of polymorphic mi-

crosatellite markers [41] indicate that most of these tumors are monoclonal. Finally, cell culture and heterotransplantation studies using cell lines established from patients with uterine CS also support the monoclonal theory of histogenesis [42–48]. These epidemiological, immunohistochemical and molecular data, together with our clinical data, suggest that the carcinomatous component of CS is dominant, akin to aggressive endometrial adenocarcinoma. CS is more aggressive than high-risk endometrial cancer, has a different pattern of spread and a worse prognosis [49]. The overall prognosis of uterine CS may be significantly worse than that of G3 endometrial adenocarcinoma and aggressive subtypes of endometrial carcinomas, such as serous and clear cell carcinomas [50]. Though a trend in this direction was observed in our study, the difference in prognosis between CS and G3 endometrial adenocarcinoma was not significant in either univariate or multivariate analyses. An explanation for this difference may lie in whether the performance of lymphadenectomy was included as a parameter in the multivariate analyses, since lymphadenectomy has been shown to affect prognosis in CS [22–24]. A surgical resection that includes lymphadenectomy improves the prognosis of CS, and when lymphadenectomy is performed, the prognosis of CS is similar to that of G3 endometrial adenocarcinoma.

Effective systemic chemotherapy for uterine CS is urgently needed. Drugs used for single-agent therapy include adriamycin [51], ifosfamide [52], cisplatin [53] and paclitaxel [54]. A recent phase II evaluation of topotecan for CS failed to demonstrate a benefit in patients with advanced or recurrent disease [55]. Two combination regimens are superior to single-agent regimens in their response rates [52, 56]. The most recent Gynecologic Oncology Group study (GOG-161) showed that for CS, the combination of ifosfamide and paclitaxel had a superior response rate and improved survival over ifosfamide alone [57]. Given the similarities between CS and endometrial carcinoma, it is logical to assume that a chemotherapeutic regimen designed for endometrial cancer would also be effective for CS [28]. Indeed, we reported that the combination of paclitaxel and carboplatin demonstrated higher activity with less toxicity than standard regimens in patients with advanced or recurrent CS [58]. Although the number of cases was small, the results of this trial argue for the conversion/metaplastic theory of CS, which behaves differently from other uterine sarcomas. Based on these results, clinical trials to confirm the effectiveness of the combination of paclitaxel and carboplatin are in progress by both the GOG (GOG-232B) and our group (Tohoku GOG).

In conclusion, our clinical results, together with previous histopathologic data, suggest that CS is distinct from pure sarcomas, bearing a stronger resemblance to epithelial tumors. Thus, treatments will be more likely to be effective if they are designed with this distinction in mind.

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SPECIAL REVIEW SERIES: Carcinogenesis of breast carcinoma and its development

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In situ production of sex steroids in human breast carcinoma

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Abstract It is well known that sex steroids are closely involved in the growth of human breast carcinomas, and the great majority of breast carcinomas express sex steroid receptors. In particular, recent studies have demonstrated that estrogens and androgens are locally produced and act in breast carcinoma tissues without release into plasma. Blockade of intratumoral estrogen production potentially leads to an improvement in the prognosis of invasive breast carcinoma patients, and, therefore, it is important to obtain a better understanding of sex steroid-producing enzymes in breast carcinoma. In this review, we summarize recent studies on tissue concentration of sex steroids and expression of enzymes related to intratumoral production of estrogens [aromatase, steroid sulfatase (STS), and 17β-hydroxysteroid dehydrogenase type 1 (17βHSD1)], and androgens (17βHSD5 and 5α-reductase) in invasive and in situ (noninvasive) breast carcinomas, and discuss the significance of intratumoral production of sex steroids in breast carcinoma.

Key words Androgen · Aromatase · Breast cancer · DCIS · Estrogen · Sex steroid

Introduction

Sex steroids, such as estrogens and androgens, play important roles in various target tissues including the reproductive organs. A majority of breast carcinoma tissues express sex steroid receptors, such as estrogen (ER), progesterone (PR), and androgen (AR) receptors,[1,2] and recent studies have demonstrated that biologically active sex steroids are locally produced and act in breast carcinoma tissues. This mechanism is considered to play a pivotal role in the proliferation of breast carcinoma cells. In particular, blockade of intratumoral estrogen production potentially reduces cell proliferation of breast carcinoma, and it is very important to obtain a better understanding of sex steroid-producing enzymes in breast carcinoma as potential therapeutic targets of endocrine therapy. Therefore, in this review, we summarize results of recent studies on tissue concentration of sex steroids and expression of sex steroid-producing enzymes in invasive and in situ (noninvasive) breast carcinomas, and we discuss the potential biological and clinical significance of intratumoral production of sex steroids in human breast carcinomas.

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In situ production of sex steroids in invasive breast carcinoma

Breast carcinoma is the most common malignant neoplasm in women worldwide, and the great majority of breast carcinoma is invasive. Among sex steroids, estrogens immensely contribute to growth of invasive breast carcinoma through binding with ER.[3] Circulating estrogens are mainly secreted from the ovary in premenopausal women, but it is also true that the majority of invasive breast carcinomas arise after menopause when the ovaries cease to be functional. Miller et al.[4] have shown that the concentration of biologically active estrogen, estradiol, was more than 10 times higher in breast carcinoma tissue than in plasma, and the intratumoral estradiol level was not significantly different between premenopausal and postmenopausal breast carcinoma patients.[5] In addition, tissue concentration of estradiol was 2.3 times higher in breast carcinoma than in the areas considered as morphologically normal.[6] Considering that invasive breast carcinomas occurring after menopause frequently express ER, local production of estrogens plays an impor-

tant role in the proliferation of invasive breast carcinoma cells in postmenopausal women.

In contrast to estrogens, androgens are considered to predominantly exert antiproliferative effects via AR in breast carcinoma cells,[7,8] although some divergent findings have been reported. Tissue concentration of androgens was investigated in invasive breast carcinomas by three groups.[9-11] Biologically active and potent androgen, 5α-dihydrotestosterone (DHT), was significantly higher in breast carcinoma tissues than in plasma,[10] and in situ production of DHT has been proposed in breast carcinoma tissues. Intratumoral DHT concentration was not significantly altered according to menopausal status in invasive breast carcinoma tissues.[11]

Figure 1 summarizes representative pathways of the local production of sex steroids in human breast carcinoma tissues, which is currently postulated. Circulating inactive steroids, such as androsteoneione and estrone sulfate, are major precursor substrates of local estrogen production. Aromatase catalyzes androstenedione into estrone, and steroid sulfatase (STS) hydrolyzes estrone sulfate to estrone. Estrone is subsequently converted to estradiol by 17β-hydroxysteroid dehydrogenase type 1 (17βHSD1) and acts locally on breast carcinoma cells through ER. On the other hand, circulating androstenedione is also converted to DHT by androgen-producing enzymes, such as 17βHSD5 (conversion from androstenedione to testosterone) and 5α-reductase (reduction of testosterone to DHT). Therefore, it is very important to examine these sex steroid-producing enzymes in breast carcinoma tissues to obtain a better understanding of the biological and clinical significance of sex steroids in breast carcinoma.

Expression of estrogen-producing enzymes in invasive breast carcinoma

Aromatase

Aromatase is an enzyme located in the endoplasmic reticulum of cells, and a single gene (CYP19) encodes aromatase in humans. Aromatase catalyzes the aromatization of androgens (androstenedione or testosterone) to estrogens (estrone or estradiol) (see Fig. 1). Aromatase is a key enzyme in estrogen biosynthesis, and aromatase inhibitors are currently used in postmenopausal patients with invasive breast carcinoma as an estrogen deprivation therapy. Approximately 70% of breast carcinoma specimens had aromatase activity comparable with or greater than that found in other tissues,[12-14] and aromatase mRNA levels

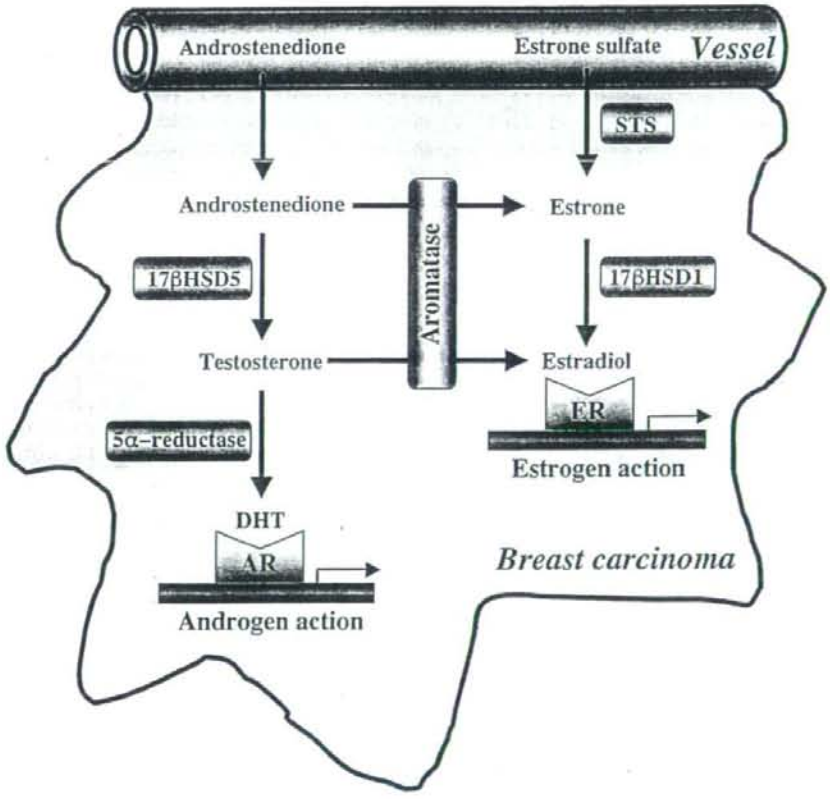

Fig. 1. Scheme representing local production of sex steroids in human breast carcinoma tissues. *STS*, steroid sulfatase; *17βHSD1*, 17β-hydroxysteroid dehydrogenase type 1; *17βHSD5*, 17β-hydroxysteroid dehydrogenase type 5; *ER*, estrogen receptor; *AR*, androgen receptor; *DHT*, 5α-dihydrotestosterone

were significantly increased in the breast carcinomas compared to those in nonmalignant tissues.[15] Aromatase was expressed in invasive breast carcinoma cells and stromal cells such as intratumoral fibroblasts and adipocytes at both mRNA and protein levels.[16] No consistent correlations between aromatase immunoreactivity and known clinicopathological factors are reported in invasive breast carcinomas.

The substrates of aromatase, i.e., androstenedione and testosterone, are not only precursors of estradiol synthesis but also precursors of DHT production (see Fig. 1). DHT itself is nonaromatizable. Intratumoral concentration of DHT was significantly associated with that of testosterone in invasive breast carcinoma tissues,[9,10] suggesting that DHT concentration in invasive breast carcinoma is possibly influenced by amount of precursor. Spinola et al.[17] showed that treatment with an aromatase inhibitor markedly elevated intratumoral testosterone concentrations in dimethylbenz (a)anthracene (DMBA)-induced rat mammary tumors, and Sonne-Hansen and Lykkesfeldt[18] reported that aromatase preferred testosterone as a substrate in MCF-7 breast carcinoma cells. In addition, very recently, Suzuki et al.[11] demonstrated that aromatase expression was inversely associated with intratumoral DHT concentration in invasive breast carcinoma tissues, and that aromatase suppressed DHT synthesis from androstenedione in coculture experiments. Therefore, aromatase is suggested to be a negative regulator of local DHT production, as well as a key enzyme of intratumoral estrogen production, in invasive breast carcinomas.

Previous in vitro studies demonstrated that breast carcinoma cells secrete various factors that induce aromatase expression in adipose fibroblasts,[19] including prostaglandin E_2,[20] interleukin (IL)-1, IL-6, IL-11, and tumor necrosis factor-α.[21,22] On the other hand, it has been also reported that exogenous growth factors such as epidermal growth factor,[23] transforming growth factor,[23] and keratinocyte growth factor[24] stimulated aromatase activity in MCF-7 cells. Very recently, Miki et al.[25] reported that mRNA level and enzymatic activity of aromatase in MCF-7 cells were significantly increased by coculture with primary stromal cells isolated from breast carcinoma tissue. Therefore, aromatase expression is suggested to be, at least in a part, regulated by tumor–stromal interactions in breast carcinoma tissues, which may be promoted by invasion of breast carcinoma into the stroma.

Other studies have demonstrated the regulation of aromatase transcription by nuclear receptors such as liver receptor homologue-1 (LRH-1)[26] and estrogen-related receptor-α (ERRα).[27] The mRNA level of aromatase was significantly associated with that of LRH-1 in adipose tissue adjacent to the invasive breast carcinoma,[26] whereas it was significantly correlated with that of ERRα in the carcinoma cells.[27] Therefore, aromatase expression in invasive breast carcinoma may be differently regulated according to the cell types.

STS

A major circulating form of plasma estrogens is estrone sulfate, a biologically inactive form of estrogen, in postmenopausal women. Estrone sulfate has a long half-life in the peripheral blood, and the level of estrone sulfate is five to ten times higher than that of unconjugated estrogens, such as estrone, estradiol, and estriol, during the menstrual cycle and in postmenopausal women.[28] STS is a single enzyme that hydrolyzes estrone sulfate to estrone (see Fig. 1).

The enzymatic activity of STS is detected in a great majority of invasive breast carcinomas, which is considerably higher than aromatase activity in breast tumors.[29] STS immunoreactivity was detected in carcinoma cells in 60%–90% of breast carcinoma cases.[30,31] STS immunoreactivity was correlated with tumor size and was significantly associated with an increased risk of recurrence in invasive breast carcinomas.[31] STS mRNA expression was also reported to be higher in breast carcinoma tissues than that in normal tissues and was significantly associated with poor clinical outcome of the patients.[32,33]

Reed et al.[34] proposed that the sulfatase pathway might be more important than the aromatase route for intratumoral estrogen synthesis in breast carcinomas, because aromatase mRNA expression was reported to have no significant prognostic value. STS inhibitors are currently being developed by several groups, and results of the phase I study suggested that STS inhibitor may be effective in hormone-dependent invasive breast carcinomas including those that progressed on aromatase inhibitors.[35]

17βHSD1

17βHSD catalyzes an interconversion of estrogens or androgens. Twelve isozymes of 17βHSD have been cloned, and 17β-reduction (17βHSD1, -3, -5, -7, etc.) or oxidation (17βHSD2, -4, -6, etc.) of estrogens and/or androgens is catalyzed by different 17βHSD isozymes. Among these isozymes, the 17βHSD1 enzyme uses NADPH as a cofactor, and mainly catalyzes the reduction of estrone to estradiol (see Fig. 1). Oxidative 17βHSD activity is the preferential direction in normal breast tissues, but the reductive 17βHSD pathway is dominant in invasive breast carcinomas.[36,37] Miyoshi et al.[5] reported that 17βHSD1 mRNA levels and intratumoral estradiol/estrone ratios were significantly higher in postmenopausal than premenopausal breast carcinomas. 17βHSD1 immunoreactivity was detected in carcinoma cells in approximately 60% of invasive breast carcinoma tissues, and it was correlated with ER and PR.[38] Therefore, it is suggested that the majority of estradiol, which is synthesized by 17βHSD1 in carcinoma cells, directly acts on these cells in breast carcinoma tissues without release into the extracellular space or plasma. Gunnarsson et al.[39] showed that breast carcinoma patients with a high level of 17βHSD1 mRNA were associated with increased risk to develop late relapse of breast carcinoma. Therefore, 17βHSD1 is considered responsible for regulating the