ANTICANCER RESEARCH *37*: 5495-5498 (2017)
doi:10.21873/anticanres.11979

Liver X Receptor (LXR)-regulated Genes of Cholesterol Trafficking and Breast Cancer Severity

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Abstract. *Background: Liver X receptor [LXR; nuclear receptor subfamily 1, group H, member 2 (NR1H2, alias LXRB)] can inhibit proliferation and induce apoptosis of cancer cells. Its relationship with disease severity is not known. Materials and Methods: Expression of LXRB, ATP binding cassette subfamily A member 1 (ABCA1), ATP binding cassette subfamily G member 1 (ABCG1), apolipoprotein E (APOE) and paraoxonase 2 (PON2) were determined in 69 breast tumors and were related to clinical stages of the disease and tumor characteristics, as well as time to recurrence. Results: ABCG1 expression differed with the tumor Scarff Bloom and Richardson (SBR) status (p=0.02), with a lower expression in SBRIII than in SBRII and SBRI. ABCG1 expression was significantly higher in estrogen receptor-positive tumors (N=63) (p=0.02). APOE expression was significantly lower in progesterone receptor-positive tumors (N=55) (p=0.03). No relationship with time to recurrence was observed. Conclusion: Expression of some LXR-dependent genes is related to breast tumor characteristics, but not time to recurrence. This may be due to a lack of study power or too short a follow-up time.*

Liver X receptors (LXRs; nuclear receptor subfamily 1, group H, member 2 (NR1H2, alias LXRB) and nuclear receptor subfamily 1, group H, member 3 (NR1H3, alias LXRA)] belong to the nuclear receptor superfamily, expressed in various cells. There are two LXR isoforms. LXRa mRNA and protein is mostly expressed in liver, kidney, intestine, adipose tissue, macrophages, lung, and spleen (1, 2), while LXRb mRNA and protein is ubiquitously expressed (3). LXRs form heterodimers with retinoid X receptor (RXR) (4) and induce genes regulating cholesterol metabolism, including the ATP-binding cassette (ABC) transporters *ABCA1* and *ABCG1* involved in cholesterol efflux from cells. LXR agonists have antiproliferative effects in various types of human cancer such as prostate (5, 6), ovarian (7) and breast (8, 9). LXRs may also enhance the production of apolipoprotein E (APOE), which inhibits the growth of several tumor cell lines, including breast carcinoma cells (10). LXR may also drive the expression of the three-gene paraoxonase (PON) family. Although data concern mainly PON1, the circulating form of this enzyme (11), it may be that all three genes are driven by LXR. Contrasting with PON1, the intracellular form of the enzyme, PON2, may prevent cell death and promote cancer (12).

However, there is a lack of data relating LXR expression and disease severity *in vivo*. The goal of our study was to relate tumor expression of some genes driven by LXR with tumor characteristics and time to recurrence in breast cancer.

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Key Words: LXR, paraoxonase, apolipoprotein E, ABCA1, ABCG1, breast cancer.

Materials and Methods

Patients and tumors. This study retrospectively included 69 good quality tumor samples from patients treated for breast cancer since 1985 at our hospital, Institut de Cancérologie de l'Ouest and preserved in Tumorothèque from the Institut de Recherche sur le Cancer Nantes Atlantique. These samples were preserved at −80˚C from the time of surgery. Data were extracted from a prospective database (Berenis®). Age at diagnosis ranged between 29 and 80 years, with a mean of 56.6 years. Informed consent was obtained from patients to use their surgical specimens and clinicopathological data for research purposes, as required by the French Committee for the Protection of Human Subjects. None had received chemotherapy, endocrine therapy or radiation therapy prior to surgery. Treatment decisions were based solely on consensus

Table I. *Expression of ATP-binding cassette subfamily A member 1 (ABCA1), ATP-binding cassette subfamily G member 1 (ABCG1), apolipoprotein E (APOE), nuclear receptor subfamily 1, group H, member 2 (LXRB) and paraoxonase 2 (PON2) genes in tumors of patients with breast cancer as defined by their clinical and tumor characteristics. Expression is given in arbitrary units as the median (5th-95th percentile).*

Factor	ABCA1	*p*-Value	ABCG1	*p*-Value	APOE	*p*-Value	LXRB	*p*-Value	PON2	*p*-Value
Stage										
0-1 (n=30)	0.83 (0.27-2.52)	0.76	1.80 (0.31-4.09)	0.65	0.68 (0.26-5.31)	0.58	0.83 (0.45-1.77)	0.27	1.81 (0.59-3.96)	0.45
2 (N=34)	0.63 (0.22-2.84)		1.76 (0.32-4.72)		1.03 (0.08-3.61)		1.03 (0.44-2.87)		2.02 (0.74-9.23)	
3-4 (N=4)	0.75 (0.60-1.20)		1.83 (1.24-3.28)		1.17 (0.49-3.70)		1.22 (0.87-1.73)		2.08 (1.78-4.44)	
N Status										
– (N=44)	0.75 (0.32-2.84)	0.51	1.84 (0.43-4.09)	0.51	0.88 (0.26-5.31)	0.40	0.83 (0.44-2.45)	0.26	1.90 (0.77-5.02)	0.73
+ (N=25)	0.68 (0.22-1.38)		1.73 (0.32-3.13)		1.02 (0.27-3.29)		1.13 (0.29-2.87)		2.12 (0.74-4.44)	
SBR*										
I (n=16)	0.79 (0.27-2.52)	0.07	1.25 (0.47-8.07)	0.02	1.05 (0.26-8.40)	0.71	0.76 (0.20-1.77)	0.60	1.51 (0.91-5.02)	0.11
II (N=42)	0.71 (0.37-2.84)		1.93 (0.66-4.09)		1.02 (0.26-3.70)		0.99 (0.45-2.87)		1.98 (0.81-8.61)	
III (N=11)	0.46 (0.20-1.29)		0.93* (0.24-3.46)		0.86 (0.24-2.23)		0.90 (0.45-2.45)		1.40 (0.72-3.24)	
PR										
– (N=13)	0.53 (0.20-8.52)	0.13	1.13 (0.24-3.46)	0.36	1.07 (0.06-6.98)	0.03	0.98 (0.20-2.45)	0.95	1.69 (0.59-9.23)	0.76
+ (N=55)	0.72 (0.27-2.52)		1.89 (0.47-4.72)		0.76 (0.26-3.61)		0.93 (0.45-2.17)		2.04 (0.74-5.02)	
ER										
– (N=5)	0.46 (0.20-1.13)	0.64	0.93 (0.31-1.38)	0.02	1.06 (0.86-2.23)	0.64	1.79 (0.45-2.92)	0.29	1.40 (0.77-3.24)	0.64
+ (N=63)	0.71 (0.27-2.52)		1.90 (0.47-4.09)		0.99 (0.26-3.70)		0.88 (0.44-2.05)		1.92 (0.74-5.02)	

SBR: Scarff Bloom and Richardson, PR: progesterone receptor, ER: estrogen receptor. *SBRIII *vs*. SBRII, p=0.02; and SBRIII *vs*. SBRI, p=0.05.

recommendations at the time of diagnosis. Overall, 90% of patients had an initial diagnosis of invasive ductal carcinoma, 65% of patients were menopausal at surgery.

RNA analysis. Nucleic acids were extracted from tumors by TRIzol (Invitrogen, Cergy Pontoise, France) as recommended by the manufacturer. After control of purity by electrophoresis, RNA (1.5 ug) was submitted to reverse transcription using SuperScript III Reverse Transcriptase (Invitrogen, Cergy Pontoise, France) as recommended by the manufacturer. An initial denaturation step of 5 minutes at 65˚C was followed by an elongation phase of 60 minutes at 50˚C and a stop reaction of 15 minutes at 70˚C. cDNA was submitted to qualitative polymerase chain reaction using ABI 7500 Fast apparatus (Invitrogen, Cergy Pontoise, France). All primer sequences were obtained from Invitrogen. Polymerase chain reaction was carried out for 40 cycles of 95˚C for 15 seconds and 60˚C for 90 seconds. The fluorescence was read continuously during the reaction and the values were normalized against three housekeeping genes: ribosomal protein large P0 (*RLP0*: Hs.546285, sequence reference: NM_001002.3), TATA box binding protein (*TBP*: Hs.590872, sequence reference: NM_001172085.1) and hypoxanthine phosphoribosyl transferase 1 (*HPRT1*: Hs.412707, sequence reference: NM_000194.2). Relative quantification was performed using the ΔΔCT method. The genes involved in cholesterol trafficking and studied in this work were *ABCA1*, *ABCG1, APOE, LXRb* and *PON2*. Gene and sequence references were as follows (gene name, unigene reference, sequence reference): ATP-binding cassette, sub-family A, member 1, Hs.659274, NM_005502.3 for *ABCA1*; ATP-binding cassette, sub-family G (WHITE), member 1, Hs.124649, NM_004915.3 for *ABCG1*; nuclear receptor subfamily 1, group H, member 2 (NR1H2), Hs.432976, NM_001256647.1 for *LXRB*; apolipoprotein E, Hs.654439, NM_000041.2 for *APOE*; and paraoxonase 2, Hs.744912, NM_000305.2 for *PON2*.

Statistical analyses. Statistical analysis was performed on SAS software, version 9.3 (Chapell Hill, NC, USA). Gene expressions were compared between groups of patients defined by clinical and tumor characteristics using the median test or the Kruskal–Wallis test (more than two groups). Curves of recurrence-free survival time from surgery were studied using the Kaplan–Meier method and risk ratios for each quartile of gene expression were calculated with quartile 1 as a reference.

Results

Results comparing groups of patients defined by their clinical and tumor characteristics are presented in Table I. Thirty patients were classified as having disease stage 0-1, 34 patients with stage 2 and four with stage 3-4 (unknown in one patient). There was no significant difference between these three groups of patients in regard to any of the genes studied. Forty-four patients had no nodal invasion at the time of surgery, while 25 patients had nodal invasion. There was no difference between these two groups of patients in regard to any of the genes studied. According to the Scarff Bloom and Richardson grading system (SBR) (13), 16 tumors were classified SBRI, 42 as SBRII and 11 as SBRIII. *ABCG1* gene expression differed significantly between these three groups (p=0.02). The between-group comparison revealed expression of *ABCG1* to be significantly lower in SBRIII

Table II. *Relative risk ratios for breast cancer recurrence within 8 years for each quartile of ATP-binding cassette subfamily A member 1 (ABCA1), ATP-binding cassette subfamily G member 1 (ABCG1), apolipoprotein E (APOE), nuclear receptor subfamily 1, group H, member 2 (LXRB) and paraoxonase 2 (PON2) gene expression in tumor. Data are risk ratios (5th-95th percentile) with quartile 1 as reference.*

Gene	Quartile	Risk ratio	*p*-Value*
ABCA1	Q2	0.56 (0.13-2.35)	0.28
	Q3	1.55 (0.49-4.90)	
	Q4	0.53 (0.13-2.21)	
ABCG1	Q2	2.10 (0.52-8.40)	0.75
	Q3	1.54 (0.34-6.85)	
	Q4	1.81 (0.43-7.58)	
APOE	Q2	1.16 (0.31-5.75)	0.20
	Q3	1.68 (0.49-5.75)	
	Q4	0.35 (0.06-1.89)	
LXRB	Q2	0.52 (0.11-2.50)	0.60
	Q3	1.56 (0.49-4.90)	
	Q4	1.02 (0.30-3.47)	
PON2	Q2	2.28 (0.67-7.75)	0.44
	Q3	1.38 (0.34-5.49)	
	Q4	0.94 (0.21-4.20)	

*Log-rank test.

compared with SBRI (p=0.05), as well as with SBRII (p=0.02). There was a tendency towards a decrease in *ABCA1* with increasing SBR grade (p=0.07). *ABCG1* was significantly higher in estrogen receptor-positive cases (p=0.02), while APOE was significantly lower in progesterone receptor-positive cases (p=0.03).

Sixty patients, with at least 8 years of follow-up since the time of surgery were included in the analysis relating gene expression and recurrence-free survival time. Eighteen cases of recurrence were recorded. As shown in Table II, no relationship was observed between gene expression and recurrence-free survival time.

Discussion

In vitro, LXR agonists reduce cancer cell proliferation and induce apoptosis (5-9). The goal of our study was to determine if the expression of *LXR* and some genes under its control in tumors would represent biomarkers of disease severity in breast cancer.

There was no relationship between stage of disease and gene expression levels. However, most patients had stage 0 to 2, while stages 3 and 4 represented a minority of the patients. Therefore, we cannot exclude the possibility that a larger number of patients with higher stages would reveal a difference. Nevertheless, when we consider the presence of nodal invasion at the time of surgery, which is another indicator of disease severity, no relationship with gene expression was observed.

Contrasting with this lack of relationship, some differences were observed between patients with different SBR grades of tumor. *ABCG1* was significantly less expressed in tumors with the highest SBR grade. Since ABCG1 protein is involved in cholesterol efflux from the cell, this fits with the initial *in vitro* observation that a lower availability of lipids to cells is associated with lower cell viability (9). In addition *ABCA1*, another gene involved in cholesterol efflux, tended to be expressed to a lesser extent in the highest SBR grade (p=0.07). *ABCG1* was also significantly highly expressed in tumors positive for the estrogen receptor, when compared with tumors negative for this receptor. It has been shown *in vitro* that estrogens induce cholesterol efflux from vascular smooth muscle cells by inducing ABCA1 and ABCG1 synthesis through LXRa activation (14). However, treatment with 17β-estradiol reduced *ABCA1* and *ABCG1* mRNA expression in estrogen receptor-positive MCF-7 cells (15). Our results suggest that activation of the estrogen pathway may induce the expression of at least *ABCG1*, one of the genes involved in cholesterol trafficking in breast tumors.

In a study describing the interaction between steroid hormones and LXR (15), it was clearly shown that treatment with progesterone reduced *LXR* expression in a progesterone receptor-positive cell model. Our results, indicating that *APOE*, a gene driven by LXR, is less expressed in progesterone receptor-positive tumors, fits perfectly with this observation. However, the absence of difference between progesterone receptor-positive and -negative tumors for the other genes driven by LXR does not argue in favor of a strong influence of this hormone on the LXR pathway in breast tumors.

The expression of LXR-activated genes in tumors was not found to be related to the recurrence-free survival time. However, our study suffers from several weaknesses. Most patients had low-stage disease, leading to a low 8-year recurrence rate. Most patients presented estrogen receptor-positive tumors. There is a crosstalk between the estrogen receptor and the LXR pathways. Although an *in vitro* study indicated that LXR activation reduces activity of estrogen and progesterone receptors (15), which may be beneficial for patients, LXR agonists may have other biological activities. It was shown that 27-hydroxycholesterol, the main LXR ligand, is also a ligand of estrogen receptor, increasing growth and metastasis of breast cancer in a mouse model (16). Although a synthetic LXR agonist reduced estrogen receptor-dependent breast cancer cell proliferation, it increased lung metastasis in the mouse model (16). Therefore, the global effect of LXR agonists derived from the diet probably reflects several contradictory influences on the disease itself.

Conclusion

It is concluded that the expression of genes driven by LXR is related to tumor grade and characteristics, but does not represent a candidate biomarker of the risk of recurrence over time.

Acknowledgements

This study was made possible by grant from the Ligue contre le cancer Loire Atlantique.

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Received July 12, 2017
Revised August 16, 2017
Accepted August 22, 2017