Expression Analysis of a Subset of Coregulators and Three Nuclear Receptors in Human Colorectal Carcinoma

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Abstract. In human colorectal tissue samples, the gene expressions of 4 coactivators, p300, pCAF, TIF-2 and TRAP 220, and 7 corepressors, N-CoR, REA, MTA1, MTA1L1, HDAC1, HDAC2 and HDAC3, linked to estrogen receptors (ER), were revealed by traditional RT-PCR. Cofactors ERα, ERβ and ERRα mRNA levels were then measured in 40 tumor tissue samples matched with respective normal mucosa by real-time PCR. The decline of mRNA levels of all coactivators and the increase of N-CoR, HDAC1, HDAC2 and MTA1 were observed from normal to tumor tissue, whereas REA, HDAC3 and MTA1L1 expressions were similar in both tissue compartments. The gene expression of ERβ correlated with those of p300, TIF-2 and REA in normal mucosa, and with that of REA in tumor tissue only. No association was found between ERα and coregulators and between each coregulator and different clinical parameters. Our findings suggest that the co-induction of ERβ and some cofactors may play an important role during the development of human colorectal carcinoma.

Coregulators are proteins that interact with ERs in a ligand-dependent manner and assemble in a multi-protein complex, thereby facilitating chromatin remodelling and regulating gene expression. The coregulators are functionally divided into two subclasses: the coactivators that enhance the ER transcriptional mechanism by their histone acetyltransferase (HAT) activity, and the corepressors that have histone deacetylase (HDAC) activity, which decreases or inhibits the ER transcriptional mechanism (6). Therefore, changes in the coregulator pattern or expression levels can affect the transcriptional activity of the estrogens and, hence, cause disorders of the ER target tissues (7).

The genomic action of the estrogens is mediated by two estrogen receptor (ER) subtypes, ERα and ERβ, that regulate growth, development and differentiation of different human tissues (1).

The definitive role of ERs in human colorectal physiology and during malignant transformation has not yet been well clarified, but recent data suggest that ERβ, rather than ERα, may play a key role in the regulation of growth and differentiation of neoplastic tissue (2-5).

Coregulators related to ERα, another nuclear receptor related to the ERα, belongs to a class of nuclear orphan receptors since no natural ligands have been discovered for it (8). The ERRα, as well as ERs, recruit several cofactors in the modulation of the ER-activated transcriptional activity (9).

Considerable progress has been made in deciphering the molecular mechanisms of the coregulators from a variety of in vitro studies, but there are few studies that have explored the correlations between ERs and their cofactors in human tissues (10-12), including human colorectal mucosa (13).

Therefore, in the first phase of our study, the presence of a series of coregulators was investigated at the mRNA level in several human colorectal tissue samples from 16 patients by the traditional reverse phase and amplification (RT-PCR) method. Since many cofactors bind the hormone-activated ERs, here, 4 coactivators [p300, pCAF (p300/CBP-Associated Factor), TIF2 (Transcriptional Intermediary Factor 2), TRAP 220 (Thyroid hormone Receptor Associated Protein)], 7 corepressors [N-CoR (Nuclear Receptor Corepressor), REA (Repressor of Estrogen receptor Activity), MTA1 (Metastasis Tumor Associated protein-1) MTA1L1 (Metastasis Tumor Associated 1-like 1)] and 3 histone deacetylases [(HDAC1, HDAC2, HDAC3)] related to ERs (7, 14) were chosen.
Subsequently, the mRNA expression levels of the same series of cofactors and nuclear receptors (ERα, ERβ, ERRα) were measured in colorectal tumor tissue samples matched with adjacent normal mucosa obtained from 40 patients by real-time PCR (QPCR). The ER and ERRα mRNA levels were correlated with those of the cofactors and, then, the coregulator mRNA levels were correlated within them. Finally, the possible associations between each cofactor expression and clinical or pathological parameters (age, sex, histological grade, TNM stage and tumor side) were also investigated.

Patients and Methods

Patients and tissue samples. A total of 40 patients from a large primary list of patients operated consecutively in our Institute was enrolled. The inclusion criteria were: (a) a negative anamnestic for familial colorectal carcinoma, (b) females patients >59 years old (women in pre- and perimenopausal period were excluded), (c) no chemo- or radiotherapy before tissue collection, and (d) no hormonal therapy in female patients.

The clinical parameters of the patients and the tumor characteristics were as follows: 27 males (median age 71 years, range 60-82), 13 females (66 years, range 56-76); 13 tumors were localized in the right colon, 7 in the left colon and 20 in the sigma-rectum. Histologically, 5 tumors were well-differentiated, 19 moderately-differentiated and 15 poorly-differentiated; 18, 14 and 8 tumors were considered to be stage II (TNM II), stage III (TNM III) and stage IV (TMN IV), respectively.

The quality of the tumor and surrounding normal tissue specimens and the relative cellular composition were determined by histopathological assessment.

All tissue specimens were stored at –80°C until evaluation of the mRNA.

Qualitative and quantitative PCR analysis. Before the PCR quantitative analysis, conventional RT-PCR analysis was performed to investigate the presence of 11 cofactor mRNAs in the colorectal tissue samples of 16 patients.

The RNA extraction and RT-PCR method have been described previously (3). In brief, 5 μl of the RT reaction, corresponding to 0.5 μg of total RNA, were subjected to the PCR in 25 μl of final volume for 45 cycles. The thermocycler program was: 30 sec at 95°C and then 10 sec at 94°C, 10 sec at 55°C and 30 sec at 72°C. The PCR products were electrophoresed in 2% agarose gel, stained with ethidium bromide, and the PCR bands were then visualized by UV.

The identity of each PCR product was confirmed by complete sequencing of the purified products. The primers used are illustrated in Table I.

Table I. Primer sequences, PCR products and published gene sequences.

<table>
<thead>
<tr>
<th>forward/reverse (f/r)</th>
<th>Primers</th>
<th>PCR product</th>
<th>GenBank</th>
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<tbody>
<tr>
<td>Coactivators</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>TIF-2 (f)</td>
<td>5’-TAATGACAGATGCTGGC-3’</td>
<td>341 bp</td>
<td>NM 006540</td>
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<tr>
<td>TIF-2 (r)</td>
<td>5’-TCTCTGATATGAGTCC-3’</td>
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<td>p300 (f)</td>
<td>5’-AGACCTGCTTCTGCTCC-3’</td>
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<td>p300 (r)</td>
<td>5’-CCAGAAGTGAAGCGGTTG-3’</td>
<td></td>
<td></td>
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<td>TRAP 220 (f)</td>
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<td>345 bp</td>
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<td>5’-TGCTCTAGACTAACAGAGG-3’</td>
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<td>Corepressors</td>
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<td>REA (f)</td>
<td>5’-CTTGAAGACATGCGGAC-3’</td>
<td>269 bp</td>
<td>AF 150962</td>
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<td>NCoR (f)</td>
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<td>HDAC2 (f)</td>
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<td>HDAC3 (f)</td>
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<td>HDAC3 (r)</td>
<td>5’-CGTACTCTGCTTGAGCGCT-3’</td>
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The primers used to detect ERα, ERβ, EREα and β-actin primers mRNAs have been published previously (3, 15). The abbreviation for each gene is explained in the text.
The QPCR methodology was essentially the same as the conventional RT-PCR described above. However, the master mix containing SYBR Green I was used (iQ SYBR Green Supermix, BioRad, Milan, Italy) and a further melt-curve step (melt-curve started at 55°C and the temperature was increased 0.5°C per cycle for 80 cycles) was added in the thermocycler program at the end of the last extension phase of 45 cycles to control the presence of the secondary non-specific PCR products or primer-dimer products that can severely reduce the amplification efficiency and sensitivity. Both qualitative and quantitative PCR reactions were performed by iCycler Thermal Cycler System apparatus (BioRad).

For quantitative analysis, the external calibration curves, one for each target gene examined and one for the β-actin gene, as the control gene, were used. Each calibration curve was performed using the amplicons of the correspondent gene transcript obtained from the conventional RT-PCR. With this approach, after purification (microClean kit; West Sussex, UK) and UV quantitation, serial dilutions of known copy number of amplicons (10²-10⁷ molecules for each PCR reaction) were used to generate a single standard curve. For each tissue sample, the absolute concentration of an unknown target gene expression was divided by that of the endogenous β-actin expression. This ratio expressed the relative mRNA level of each target gene.

Statistical analysis. The relative gene expression levels between tumor tissue and surrounding normal mucosa were tested using the two-tailed Wilcoxon signed-rank.

The Spearman’s rank correlation was used to study the relationships between two different gene mRNA levels, and the non-parametric Kruskal-Wallis test was used for the associations between each gene expression level and clinical or tumor parameters.

The relative mRNA levels were expressed as mean±SEM, and two-sided p≤0.05 was considered as statistically significant.

Results

RT-PCR analysis. Conventional RT-PCR analysis confirmed the presence of all cofactors examined. The results obtained are shown in Figure 1.

Results of the QPCR optimization. The real-time PCR efficiencies of the standard curves were obtained from the given slopes using the formula: E=10(-1/slope) (iQ Thermal
Cycler software, BioRad). The PCR efficiencies, expressed in percent, ranged from 92% to 98% for nuclear receptors, cofactors and β-actin (data not shown).

To confirm the accuracy and reproducibility of the QPCR, the intra-assay precision, determined in three PCR runs of the same RNA sample, was <2.5%. Inter-assay variation, investigated in 3 different experimental runs performed on 3 days using the aliquots of a pool of the total RNA, was <3.7%.

Expression analysis of 11 cofactors and 3 nuclear receptors in colorectal tissue samples by QPCR. The distributions of the nuclear receptor and cofactor mRNA levels, respectively, in tumor tissues and in adjacent normal mucosa of a well-defined cohort of 40 patients, are shown in Figures 2A and 2B.

The ERα and ERβ expressions were lower in malignant tissue samples than in non-malignant epithelium \( (p=0.019 \text{ and } p=0.029, \text{ respectively}) \), whereas the ERαt expression was found to be higher in the colorectal tumor tissue than in normal mucosa \( (p=0.009) \). These data are in accordance to those previously published (15).

Among the coactivators, the pCAF, TIF-2, p300 and TRAP-220 mRNA levels were lower in cancerous tissue than in adjacent normal mucosa \( (p=0.048, p=0.047, p=0.013, \text{ TIF-2; } p=0.040, \text{ TRAP-220}) \). Among the corepressors, the mRNA expression levels of NCoR, HDAC1, HDAC2 and MTA1 were higher in the carcinomas than in the normal colorectal mucosa, but statistical significance was reached only for HDAC2 and MTA1 mRNA levels \( (p=0.029, p=0.024, \text{ respectively}) \).

The expression profile of the other corepressors (REA, HDAC3 and MTA1L1) did not change between malignant and normal colorectal tissues.

Relationships within gene expression levels. The possible relationships between coregulator mRNA levels and those of ERα, ERβ and ERαt, either in tumor tissue or in adjacent normal mucosa, were analyzed.

a) Colorectal normal mucosa: The ERβ expression was significantly related with TIF-2 \( (r=+0.64, p=0.006), \) p300 \( (r=+0.56, p=0.0042) \) and REA \( (r=+0.44, p=0.047) \) expressions. An inverse correlation was observed between ERαt and TRAP 220 \( (r=-0.51, p=0.044) \) mRNA levels, and no significant associations were observed between ERαt and each coregulator gene expression.

In addition, the following correlations were observed between coactivators and corepressors: TIF-2 (coactivator) and REA (corepressor; \( r=+0.53, p=0.030 \)), TIF-2 and HDAC1 \( (r=-0.73, p=0.020) \) and a trend toward significance was observed between TIF-2 and HDAC2 \( (r=-0.58, p=0.090) \). A positive correlation was also found between HDAC2 and HDAC1 mRNA levels \( (r=+0.60, p=0.040) \).

b) Colorectal tumor tissue: In this tissue compartment, the ERβ expression correlated only with that of the corepressor REA \( (r=+0.61, p=0.004) \), whereas the ERαt or ERαt mRNA levels and each cofactor expression were not significant. Finally, an association between HDAC1 and HDAC2 expressions \( (r=+0.59, p=0.032) \) was observed.

The gene expression levels among patient subgroups defined by clinical and pathological parameters (age, sex, tumor side, histological differentiation and TNM grade) did not show any association (data not shown).

Discussion

The transcriptional activity of the ERs and other nuclear receptor superfamily members, such as ERRαt, is modulated by different coregulator proteins.

Although we can begin to understand the critical role of ER-mediated signaling in the development and progression of human colorectal carcinoma, the role of altered expression of the ER coregulators is still elusive for this tissue. For this reason, our first set of experiments was designed to elucidate, at the mRNA level, whether a subset of the cofactors was present in human colorectal tissue, as already found in other human tissues.

The qualitative PCR analysis revealed the presence of all cofactors examined. The presence of these cofactors in the colorectal tissue as well suggests that the cofactors can be considered ubiquitous in the human organism.

However, the changes in the expression levels of these cofactors along with the changes in the ER expressions might be important in determining the tissue-specific response to a particular steroid hormone (10). Therefore, in the second series of experiments, the expression levels of the ER cofactors and 3 nuclear receptors were screened in human colorectal tumor tissue matched with adjacent normal mucosa samples obtained from 40 patients. Even if the small sample number \( (n=40) \) of patients may limit the statistical power of the analyses, we have demonstrated the significant overexpression of ERRαt and the up-regulation of 2 ERs from normal to tumor tissue, as previously found with a higher number of patients (15).

The same QPCR analysis also showed the decline of the coactivator and the increase of the corepressor mRNA levels from normal to tumor tissue and a similar amount of some corepressors in both tissue compartments. These data suggest a different role of the coactivators and corepressors in normal and in tumor colorectal tissues, respectively, and it seems that the coactivator action predominates in the normal tissue compartment, whereas that of the corepressors in pathological tissue. However, the validity of this hypothesis should be tested in more extensive
experiments at the protein level or in in vitro experiments.

Despite the small number of cofactors examined, approximately 50 cofactors have been discovered in human tissues, and the following associations within gene expression have been found. The ERβ correlated with TIF2 and p300 (coactivators) and with REA (corepressors) in normal tissue, whereas the tumor TIF2 expression was associated only to tumor REA expression. Since the ERβ transcriptional activity can be modified by selective ER modulators (coactivators and/or corepressors), our data suggest that the ER target gene expression levels may be modulated by a common regulatory pathway that expresses a balance between enhancement and suppressive effects. This is in accordance with other authors (14) who, for the first time, suggested a synergistic role between ERs and cofactors in the development and progression of human breast cancer.

In our study, the HDACs seem not to be involved in the regulation of the ER target genes in the normal colorectal tissue compartment, since we found an inverse correlation between TIF2 and HDAC1 or HDAC2 expressions. The corepressors, such as HDACs, contain the histone deacetyltransferase activity that maintains the ER target genes in an inactive state, and this condition is generally more expressed in pathological than in normal tissues (16). Another association was found between HDAC1 and HDAC2 mRNA levels, either in the tumor tissue or in the surrounding normal mucosa. It has been shown recently that these 2 HDACs have a high degree of homology and coexist within the same protein complexes on the DNA structure during the translation process, even if the individual HDAC might function in a distinct manner (17).

The lack of association between ERα expression and cofactors suggests that the ERβ, rather than ERα, is the principal ER subtype in the estrogenic transcriptional activity in the human colorectal tract, but it is also possible that other cofactors are involved in the activated-ERα activity. The ERα and ERβ are not equally potent transcriptional activators on the chromatin structure, and although both ERα and ERβ bound equally well to an ERE (Estrogen Responsive Element) sequence present in the promoter of the ER target genes, the differences between the 2 ERs in their transcriptional activities may be due to a different receptor-dependent chromatin remodeling (18).

Finally, since there is a lack of information on the functional role of ERRα in human colorectal mucosa, the importance of the inverse association between ERRα and TRAP 220 mRNA levels in normal tissue is still unknown.

In conclusion, our study, the first exploratory analysis on the coregulator expression in the human colorectal tract, revealed a co-induction of ERβ and several cofactors that may influence the transcriptional machinery of the ER target genes or may play an important role in the development and progression of human colorectal carcinoma.

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References


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