Molecular Characteristics of ERCC1-Negative versus ERCC1-Positive Tumors in Resected NSCLC

Luc Friboulet1,2,7, Daniel Barrios-Gonzales2,3,7, Frédéric Commo1,2,7, Ken André Olaussen1,2,7, Stephan Wagner1,2,7, Julien Adam1, Aïcha Goubar1,2,7, Nicolas Dorvault1,2,7, Vladimir Lazar5, Bastien Job9, Benjamin Besse2,3,7, Pierre Validire6, Philippe Girard6, Ludovic Lacroix1,4, Johanna Hasmats5, Fabienne Dufour5, Fabrice André1,2,3,7, and Jean-Charles Soria1,2,7

Abstract

**Purpose:** Excision repair cross-complementation group 1 (ERCC1) is a protein involved in repair of DNA platinum adducts and stalled DNA replication forks. We and others have previously shown the influence of ERCC1 expression upon survival rates and benefit of cisplatin-based chemotherapy in patients with resected non–small-cell lung cancer (NSCLC). However, little is known about the molecular characteristics of ERCC1-positive and ERCC1-negative tumors.

**Experimental Design:** We took advantage of a cohort of 91 patients with resected NSCLC, for which we had matched frozen and paraffin-embedded samples to explore the comparative molecular portraits of ERCC1-positive and ERCC1-negative tumors of NSCLC. We carried out a global molecular analysis including assessment of ERCC1 expression levels by using both immunohistochemistry (IHC) and quantitative reverse transcriptase PCR (qRT-PCR), genomic instability, global gene and miRNA expression, and sequencing of selected key genes involved in lung carcinogenesis.

**Results:** ERCC1 protein and mRNA expression were significantly correlated. However, we observed several cases with clear discrepancies. We noted that ERCC1-negative tumors had a higher rate of genomic abnormalities versus ERCC1-positive tumors. ERCC1-positive tumors seemed to share a common DNA damage response (DDR) phenotype with the overexpression of seven genes linked to DDR. The miRNA expression analysis identified miR-375 as significantly underexpressed in ERCC1-positive tumors.

**Conclusions:** Our data show inconsistencies in ERCC1 expression between IHC and qRT-PCR readouts. Furthermore, ERCC1 status is not linked to specific mutational patterns or frequencies. Finally, ERCC1-negative tumors have a high rate of genomic aberrations that could consequently influence prognosis in patients with resected NSCLC. *Clin Cancer Res;* 17(17); 5562–72. ©2011 AACR.
Translational Relevance

Adjuvant chemotherapy has a significant, but limited effect in prolonging survival. The use of biomarkers to select patients and optimize treatment options is essential. Excision repair cross-complementation group 1 (ERCC1) is a promising biomarker, but the ID-card of ERCC1-positive versus ERCC1-negative tumors is unclear.

To better characterize ERCC1-positive versus ERCC1-negative tumors, we carried out a global molecular analysis on 91 resected non–small-cell lung cancer samples. This study addresses some of the current debates in the literature, notably it carries out a comparison between immunohistochemistry (IHC) and quantitative reverse transcriptase PCR to evaluate ERCC1 expression level and estimates the link between cancer-gene mutations (i.e., epidermal growth factor receptor) and ERCC1 status.

The differences between ERCC1-negative and ERCC1-positive tumors might help to apprehend the underlying mechanisms of chemoresistance. Our data suggest that ERCC1 status could be associated with higher genomic instability with a specific pattern of DNA damage response gene expression. Furthermore, miRNA expression may explain the discrepancies between IHC and mRNA measurements of ERCC1.

repair, these adducts interfere with DNA transcription and replication, eventually inducing cell death (6). Therefore, DNA damage repair pathways, in addition to their implications in genomic instability and tumorigenesis, are highly relevant to predict platinum resistance (7). There are at least 6 DNA repair pathways known to correct DNA damage: direct repair, base excision repair (BER), nucleotide excision repair (NER), mismatch repair, homologous recombination repair, nonhomologous end joining. The mechanism of repair mobilized depends on the type of DNA damage and the cell-cycle phase.

The excision repair cross-complementation group 1 (ERCC1) gene encodes a key enzyme in the NER pathway, which recognizes and removes platinum DNA adducts (8). The ERCC1/XPF (xeroderma pigmentosum complementation group F) heterodimer is responsible for incising DNA at the 5’ site of the lesion during NER. ERCC1/XPF is also involved in the repair of platinum-induced DNA interstrand crosslinks (ICL) to allow the release of stalled replication forks.

We and others have showed that ERCC1 expression is both a prognostic and predictive marker in NSCLC (9, 10). ERCC1-negative expression in tumors is predictive of survival benefit from cisplatin-based adjuvant chemotherapy, whereas in the absence of therapy, patients with ERCC1-negative tumors have a worst prognosis.

ERCC1 value has also been explored in gastric cancer (11), esophageal cancer (4), ovarian cancer (12), head and neck cancer (13), bladder cancer (2), and colorectal cancer (14). In these tumors, a high level of ERCC1 mRNA or protein expression correlated with poor overall survival and resistance to platinum-based chemotherapeutic treatments. Nevertheless, some studies have not been able to correlate ERCC1 expression level with survival (15, 16). Both immunohistochemistry (IHC) and quantitative reverse transcriptase PCR (qRT-PCR) are usually used to assess ERCC1 expression. A correlation between ERCC1 mRNA (qRT-PCR) and protein expression (IHC) has been observed in one cohort (17), but not in others (18). Thus, the choice of one technique to the other could explain the discrepancies between different studies.

Several studies have also investigated whether or not the presence of EGFR mutation was related to the prognosis of resected NSCLC (3, 19). Interestingly, EGFR-activating mutations were associated with low ERCC1 expression both at the mRNA (20) and protein level (19). This is in contrast with previous studies that suggested that ERCC1 expression was upregulated by EGFR pathway activation (21, 22).

Little is known about the specific molecular differences between ERCC1-positive versus ERCC1-negative tumors. Therefore, we aimed at obtaining a better picture of the molecular background associated with a specific ERCC1 status. To identify molecular differences between ERCC1-positive and ERCC1-negative NSCLC tumors, we carried out a global molecular analysis on a cohort of 91 resected NSCLC patients for which we had matched frozen and paraffin-embedded tissues. We analyzed the correlation between ERCC1 IHC status and (a) ERCC1 mRNA expression level (qRT-PCR), (b) genomic instability (CGH), (c) global gene expression (transcriptome analysis microarrays), (d) miRNA expression (miRNA microarrays), and (e) sequencing of important genes involved in lung carcinogenesis (TP53, KRAS, EGFR, PIK3CA, BRAF, ERBB2, KDR, AKT1, and ERCC1).

Materials and Methods

Patients and samples

NSCLC patients underwent surgical resection in a single institution (Institut Mutualiste Montsouris in Paris, France) between 2002 and 2006. The key inclusion criterion was the availability of matched formalin-fixed paraffin-embedded (FFPE) and frozen samples. The present cohort belongs to the CHEMORES initiative (Chemotherapy resistance consortium), which is an EU-funded (FP6) research collaboration involving 19 universities, organizations for cancer research, and research-oriented biotechnology companies in 8 European countries.

ERCC1 immunohistochemistry

FFPE tissue sections were stained with mouse monoclonal antibody against ERCC1 (ab-2, clone 8F1, ref: MS-671-P; Thermo scientific), as previously described (9). Microscopic evaluation of the ERCC1 staining was carried out independently by 3 observers (D.B., K.A.O., and J.A.)
in a blinded fashion. The staining intensity was graded on a scale of 0 to 3 based on the intensity of internal control cells (endothelium, bronchial epithelium, or fibroblast cells close to the tumor) that was systematically assigned an intensity of 2. The percentage of positive nuclei was estimated for each specimen, and a proportional score was assigned (0 if 0%, 0.1 if 1%–9%, 0.5 if 10%–49%, and 1 if 50% or more). The proportional score was multiplied by the staining intensity to obtain a final semi quantitative H Score, as previously described (9).

RNA extraction and qRT-PCR
No macrodissection or microdissection of the tissues before mRNA extraction was carried out. The lysis of each NSCLC frozen tissue sample was obtained by using a polystyrene homogenizer (ultraturrax, IMLAB). The RNA extraction was carried out with TRIzol Reagent Protocol (Invitrogen). Total RNA was quantified and qualified with Nanodrop ND-1000 spectrometer and Bioanalyser-2100 (Agilent). The primers and probe sequences used for the qPCR for ERCC1 were the following: forward primer: 5’-GGGAATTTGGCGACG- TAAITC-3’, reverse primer 5’-GGGAAGCCTGGAACAG- 3’, probe 6FAM 5’-CAGAGCTCCTGGCCACACAGATA- TAM-3’. For PPIA: 5’-GTCACCCCCACGGTGTCT-3’, 5’- CTGCCGCTCTTGGCCACAGT-3’, 6FAM 5’-AGTCGAAAG- GAGACGGGGCCTC-3’, for the forward, reverse primer, and probe, respectively. The ERCC1 primers recognize exon 5 of the ERCC1 gene common to all known ERCC1 isoforms. The relative expression of ERCC1 mRNA was determined by using the C\(_t\) value and the 2\(^{-}\Delta\Delta C\(_t\)\) method. The data are presented as the fold change in gene expression normalized to an endogenous reference gene (PPIA) and relative to a human reference CDNA.

CGH
The DNA copy number profiling was carried out by using dual-color competitive hybridization on 244K Whole Human Genome (G4411B) microarrays (Agilent Technologies). In each case, the normal tissue sample was used as the reference to its corresponding tumor sample. Tumor aberration rates were defined as the ratio between the total length of aberrations in a given tumor sample versus the full size of the human genome as defined by the UCSC genome build hg18. See Supplementary methods for experimental procedures (23).

Gene expression microarrays
Gene expression profiling was carried out by using dual-color 244K Human Exon Array (design-custom) 24891 from Agilent. Each normal sample was used as the reference to its corresponding tumor sample. RNA extractions, Cy3 (normal samples) and Cy5 (tumor samples) labeling, and hybridizations were carried out according to Agilent’s instructions.

Slides were scanned with an Agilent Microarray Scanner G2505B (Agilent Technologies Inc.), and Feature Extraction normalized data were imported into R software. See Supplementary methods for analysis details.

miRNA microarrays
The miRNA expression profiling of tissue samples was carried out on Agilent Human microRNA microarray v3.0. According to Agilent’s recommendations, normal and tumor samples were hybridized separately. Briefly, isolated total RNA was dephosphorylated with calf intestinal phosphatase, labeled with pCp-Cy3 and T4RNA ligase, and hybridized for 20 hours at 55°C. Slides were washed and scanned with an Agilent MicroArray Scanner G2505B. Feature Extraction data were imported into R software, then quantiles normalized, and log\(_2\) transformed before statistical analysis.

Gene mutations analysis
A search for mutations by sequencing of 9 target genes was carried out internally by the translational research laboratory at Institut Gustave Roussy (Villejuif, France), except for p53 status which was analyzed by the Royal Institute of Technology (Stockholm, Sweden). We analyzed full coding sequences of exons including oncogenic mutational hot-spots in NSCLC corresponding to: TPS3 (NM_000546.4) exons 5–8; Kras (NM_004448.2) exons 2, 3; EGRF (NM_005228.3) exons 18–21; Pik3ca (NM_006218.2) exons 10, 21; Braf (NM_004333.4) exon 15; Erbb2 (NM_004448.2) exons 18, 20–24; Kdr (NM_002253.1) exons 2, 26, 27, 30; Akt1 (NM_005163.2) exon 4, and Ercc1 (NM_202001.1) all exons. See Supplementary methods for experimental procedures.

Statistical analysis
All statistical analyses were conducted with R software. A SAM procedure (R software, samr package) was carried out to define genes or miRNA differentially expressed in tumors regarding their ERCC1-IHC status. A Q value (%) of 5 of less was considered as significant. For CGH, frequencies of gain, or loss, were tested for their association with the ERCC1-IHC status using a Pearson’s χ\(^2\) test using a Monte Carlo approximation on 2,000 replicates. P values were then corrected with the Benjamini–Hochberg method (24). A P value (%) of 5 or less was considered as significant. CGH aberration rates and qRT-PCR values were considered as continuous values and were compared regarding ERCC1 IHC status with a Wilcoxon test. The Kaplan–Meier method was used to estimate the overall survival curves according to CGH aberration rate. For this, patients were assigned into low or high aberration rate group by the k-means clustering method. A log-rank test was used to compare rates between groups.

Results
Clinicopathologic characteristics
Among 121 initial tumors stained for ERCC1, 14 were excluded from further analysis because of invalid staining of internal control cells during IHC staining, and 16 were excluded because they contained less than 50% of tumor cells. All molecular and statistical analyses were thus...
conducted on the 91 remaining samples. The clinicopathologic characteristics of the 91 included patients are shown in Table 1. The median age was 63 years. Patients were more frequently men in both ERCC1-positive and ERCC1-negative groups (70%–73%). Pathologic stages were stage I in 46 cases (52%), stage II in 18 cases (20%), and stage III in 25 cases (28%). In the ERCC1-negative group, tumors were predominantly adenocarcinomas (61%), whereas in the ERCC1-positive group, there was a majority of squamous cell carcinomas (SCC; 60%, \( P = 0.001 \)) as previously reported (9). Almost half of the patients received adjuvant chemotherapy, and 88% did not receive adjuvant radiotherapy.

Table 1. Patients’ clinicopathologic characteristics

<table>
<thead>
<tr>
<th></th>
<th>All patients, ( n = 91 )</th>
<th>Patients with ERCC1 IHC-negative tumors, ( n = 41 )</th>
<th>Patients with ERCC1 IHC-positive tumors, ( n = 50 )</th>
<th>( P^a )</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Gender</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>26 (28.6)</td>
<td>11 (26.8)</td>
<td>15 (30.0)</td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>65 (71.4)</td>
<td>30 (73.2)</td>
<td>35 (70.0)</td>
<td>0.92</td>
</tr>
<tr>
<td>Ageb median (range)</td>
<td>62.9 (40.9–84.7)</td>
<td>61.4 (45.3–84.7)</td>
<td>63.3 (40.9–81.8)</td>
<td>0.54</td>
</tr>
<tr>
<td><strong>Stage</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>46 (51.7)</td>
<td>19 (46.3)</td>
<td>27 (56.3)</td>
<td></td>
</tr>
<tr>
<td>II</td>
<td>18 (20.2)</td>
<td>10 (24.4)</td>
<td>8 (16.7)</td>
<td></td>
</tr>
<tr>
<td>III</td>
<td>25 (28.1)</td>
<td>12 (29.3)</td>
<td>13 (27.1)</td>
<td>0.57</td>
</tr>
<tr>
<td>Unknown</td>
<td>2</td>
<td>0</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td><strong>TNM status</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T: 1</td>
<td>11 (12.2)</td>
<td>3 (7.3)</td>
<td>8 (16.3)</td>
<td></td>
</tr>
<tr>
<td>T: 2</td>
<td>62 (68.9)</td>
<td>30 (73.2)</td>
<td>32 (65.3)</td>
<td></td>
</tr>
<tr>
<td>T: 3</td>
<td>10 (11.1)</td>
<td>5 (12.2)</td>
<td>5 (10.2)</td>
<td></td>
</tr>
<tr>
<td>T: 4</td>
<td>7 (7.8)</td>
<td>3 (7.3)</td>
<td>4 (8.2)</td>
<td>0.62</td>
</tr>
<tr>
<td>Unknown</td>
<td>1</td>
<td>0</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>N: 0</td>
<td>50 (56.2)</td>
<td>23 (56.1)</td>
<td>27 (56.3)</td>
<td></td>
</tr>
<tr>
<td>N: 1</td>
<td>21 (23.6)</td>
<td>11 (26.8)</td>
<td>10 (20.8)</td>
<td></td>
</tr>
<tr>
<td>N: 2</td>
<td>18 (20.2)</td>
<td>7 (17.1)</td>
<td>11 (22.9)</td>
<td>0.70</td>
</tr>
<tr>
<td>Unknown</td>
<td>2</td>
<td>0</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td><strong>Smoking status</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Current or former</td>
<td>87 (96.7)</td>
<td>39 (97.5)</td>
<td>48 (96.0)</td>
<td></td>
</tr>
<tr>
<td>Never</td>
<td>3 (3.3)</td>
<td>1 (2.5)</td>
<td>2 (4.0)</td>
<td>0.70</td>
</tr>
<tr>
<td>Unknown</td>
<td>1</td>
<td>1</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td><strong>Histology</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Adenocarcinoma</td>
<td>40 (44.0)</td>
<td>25 (61.0)</td>
<td>15 (30.0)</td>
<td></td>
</tr>
<tr>
<td>SCC</td>
<td>38 (41.8)</td>
<td>8 (19.5)</td>
<td>30 (60.0)</td>
<td></td>
</tr>
<tr>
<td>Other</td>
<td>13 (14.3)</td>
<td>8 (19.5)</td>
<td>5 (10.0)</td>
<td>0.001</td>
</tr>
<tr>
<td><strong>Adjuvant chemotherapy</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>41 (45.1)</td>
<td>13 (31.7)</td>
<td>28 (56.0)</td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>50 (54.9)</td>
<td>28 (68.3)</td>
<td>22 (44.0)</td>
<td>0.04</td>
</tr>
<tr>
<td><strong>Adjuvant radiotherapy</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>80 (87.9)</td>
<td>36 (87.8)</td>
<td>44 (88.0)</td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>11 (12.1)</td>
<td>5 (12.2)</td>
<td>6 (12.0)</td>
<td>0.98</td>
</tr>
</tbody>
</table>

Abbreviation: TNM, tumor-node-metastasis.

For categorical data, \( \chi^2 \) test or fisher exact when required and Wilcoxon signed-rank test for continuous data.

Age at surgery.

Patient classification according to IHC assessment of ERCC1 expression

The median percentage of cells with positively stained nuclei was 60% (range 0% to 90%), whereas the median value of intensity was 2. Tumors with an H-score exceeding 1.5 were considered ERCC1 positive as previously reported (9). Among the 91 tumors, 50 were classified as positive and 41 as negative. Figure 1A displays an example of ERCC1 nuclei staining with a positive staining intensity of 3. In this small cohort, ERCC1 protein expression level had no prognostic or predictive value (data not shown). ERCC1 status reported in the rest of this manuscript was defined by IHC unless otherwise specified.
Correlation between ERCC1 mRNA (qRT-PCR) and protein expression levels (IHC)

We compared ERCC1 H-scores determined by IHC to the relative amount of all known ERCC1 mRNA transcripts determined by qRT-PCR. ERCC1 IHC status was significantly associated with the relative amount of ERCC1 mRNA transcripts (Fig. 1B). The median of ERCC1 $2^{-\Delta\Delta C_t}$ was 0.75 in the ERCC1 IHC-negative group compared with 1.06 in the ERCC1 IHC-positive group ($P = 0.003$). A logistic regression model confirmed the correlation between the relative expression of ERCC1 mRNA and the probability of ERCC1 IHC-positive status ($P = 0.004$, Fig 1C). The median mRNA expression (0.92) was chosen as a cutoff point to identify patients as “low ERCC1” or “high ERCC1” (as previously described; ref. 25). Figure 1D illustrates the spectrum of ERCC1 mRNA expression levels, centered on the global median, according to ERCC1 IHC status with a significant difference between both groups ($\chi^2, P < 10^{-3}$). Of note, Figure 1D suggests that this correlation is far from being perfect, because 33% of the samples were ERCC1 low by qRT-PCR in ERCC1 IHC-positive tumors. In ERCC1 IHC-negative tumors, 32% of the samples were high for ERCC1 by qRT-PCR. We therefore investigated whether these disparities could be explained by the presence of ERCC1-targeted miRNA differentially expressed between ERCC1-positive and ERCC1-negative tumors.

Differential miRNA expression according to ERCC1 status

We carried out a global miRNA expression analysis. The best patient classification regarding ERCC1-IHC status was
obtained by considering the first 29 miRNA when ordered by Q values (Fisher exact test, \( P = 3.10^{-3} \) on 29 miRNA, Fig. 2A and Supplementary Table S1). When selecting Q values lower than 10\(^{-3}\) for each individual miRNA, 4 were expressed differentially between the 2 groups. Among these, hsa-miR-375 was the most differentially expressed with a fold change of \(-3.3\) in the positive group compared with the negative one (Fig. 2B). None of these 4 miRNA has a predicted interaction with ERCC1 mRNA according to TargetScan, release 5.1 (http://www.targetscan.org). With other target prediction program (miRGen: http://www.diana.pcbi.upenn.edu/miRGen.html), we identified, among the genes predicted to be targeted by miR-375, 9 genes linked to DNA repair: TP53, USP1, APEX1, TYMS, MLH3, XRCC6BP1, ERCC3, NTHL1, and PARP4.

However, when searching exclusively for the presence of ERCC1-targeting miRNA that could explain disparities between ERCC1 protein and mRNA expression levels, we identified hsa-miR-409-3p. Indeed, the expression of miR-409-3p increased in parallel with ERCC1 mRNA expression specifically in ERCC1-negative tumors (Fig. 2C), whereas, it had a stable expression in ERCC1-positive tumors. miRNA can decrease the protein expression by adapting its own expression to mRNA expression of the target gene. Here, miR-409-3p could maintain low ERCC1 protein expression in ERCC1-negative tumors even when ERCC1 mRNA expression increased. This phenomenon can contribute to the observed discrepancies between IHC and qRT-PCR results.
ERCC1 expression influences genomic aberration rates (CGH)

The DNA copy number profiling showed some weak differences in gain/loss frequencies between the 2 ERCC1 IHC statuses. Precisely, we observed higher frequencies of gains in 8q and 14q regions in ERCC1 IHC-positive tumors. But both seemed nonsignificant after P value correction (Supplementary Fig. S1).

However, the median of aberration rates [defined as the sum of lengths of aberrations related to the size of explored genome, for each tumor sample (categorized either as a relative gain or loss of DNA copy number); Fig. 3A] was 43.2% in the ERCC1-negative group (range from 0.02 to 78.5) compared with 24.2% in the ERCC1-positive group (range 0.05–72.4; Fig. 3B). This difference in rates, regarding ERCC1 IHC status, appeared significant ($P = 0.004)$ and suggests a higher genetic instability among ERCC1-negative tumor samples as compared with positive ones. Interestingly, the analysis on overall survival according to the overall rate of genomic aberrations in the tumors (CGH) suggested that CGH aberration rate was not associated with survival ($P = 0.47$), but high aberrant tumors clearly tended to have a worst prognosis (Fig. 3C).

ERCC1 protein expression is not associated with a specific pattern or frequency of mutations in relevant cancer-related genes

We then examined if ERCC1 IHC status reflected genomic instability in terms of number of mutations. We analyzed the number of mutations by direct sequencing of mutational hotspots in 9 key genes in NSCLC (EGFR, KRAS, TP53, PI3CA, BRAF, AKT1, KDR, ERBB2, and ERCC1). Several mutations were detected, but no significant differences were observed between ERCC1-positive and -negative groups (Fig. 3D). Only one mutation in ERCC1 exon 8 was found. Concerning EGFR, 6 activating mutations were identified and for TP53 we found mutations in 27% of patients. The details of mutations detected are presented in Supplementary Table S2 (26).

Figure 3. ERCC1 expression influences genomic aberration rates. A, example of a chromosome 9’s genomic profile given by CGH, the genomic aberrations are shown in red (amplifications) and green (losses), and the thresholds of significance are shown by the dotted yellow line. To calculate the percentage of total aberrant genome per sample, we divided total aberrant megabases by total analyzed genome size in each patient. B, boxplot repartition of ERCC1 genomic aberration rates according to ERCC1 IHC status. C, overall survival Kaplan–Meier curve according to CGH aberration rate in tumors. D, ERCC1 status and mutations analysis on 9 cancer genes: TP53 (NM_000546.4) exons 5–8; KRAS (NM_004448.2) exons 2, 3; EGFR (NM_000228.3) exons 18–21; PIK3CA (NM_006218.2) exons 10, 21; BRAF (NM_004333.4) exon 15; ERBB2 (NM_004448.2) exons 18, 20–24; KDR (NM_002253.1) exons 2, 26, 27, 30; AKT1 (NM_005163.2) exon 4, and ERCC1 (NM_202001.1) all exons.
Differential gene expression between ERCC1-positive and -negative tumors

We carried out a global transcriptome analysis to evaluate whether there were any significant differences in gene expression between ERCC1-positive and -negative tumors. We found 149 genes that were differentially expressed between the 2 groups (Fig. 4A and Supplementary Table S3). None of these genes have previously been described to belong to the NER pathway or any of the other DNA repair pathways. However, we found 7 genes strongly associated with DNA damage response (DDR), and all were increased in the ERCC1-positive group. These DDR-related genes were CCNA1, KLF4, MYC, RNF168, S100A2, SNAI2, and TP53AIP1 (GO: 0006974; Fig. 4B). Three genes overexpressed in ERCC1-positive tumors are linked to the EGFR signaling pathway ITGA6, KRT16, and KRT6B (GO:0007173).

Discussion

One of the major challenges in current cancer chemotherapeutic treatment is the identification of effective biomarkers for drug response prediction (27). Although many studies suggested that ERCC1 is a potential prognostic and predictive marker, there is no consensus on the optimal method for ERCC1 evaluation (IHC vs. qRT-PCR), and little is known regarding the molecular basis related to the intrinsic prognostic value of ERCC1. Here, we carried out a "system biology" approach including CGH arrays, gene expression arrays, miRNA microarrays, and gene sequencing analysis in 91 resected NSCLC to better apprehend how ERCC1 IHC-positive tumors might be different from their negative counterparts.

In several malignancies, ERCC1 mRNA expression has shown to be related with clinical outcome and response to DNA damaging chemotherapy (28). Metzger and colleagues reported increased ERCC1 mRNA expression as an indicator for nonresponse to neoadjuvant cisplatin-based chemotherapy (29). However, they did not study the correlation between ERCC1 expression at the protein and transcriptional level. We found a certain degree of association between ERCC1 mRNA levels and ERCC1 protein expression observed by IHC. This association was somehow imperfect, and the overlap between the populations was considerable. In line with our results, Doll and colleagues have described a similar degree of correlation between ERCC1 mRNA and protein expression, with clear areas of discrepancies (17). Another study was unable to correlate ERCC1 mRNA and protein expression (18). These disparities can be explained by technical reasons. Indeed, the IHC approach takes into account the percentage and intensity of protein expression directly in tumor cells. On the contrary, the qRT-PCR depends on the quality of the sample (RNA is easily altered), and the percentage of tumor cells in the sample varies from one sample to another. It should be noted that no macrodissection or microdissection of the tissues was carried out in our samples before mRNA extraction. Biological reasons can also contribute to the observed discrepancies. Considering the importance of the posttranscriptional regulatory mechanisms of ERCC1 expression, the mRNA expression level could be very distant from the protein level. Indeed, we observed that an increase in ERCC1 mRNA expression was not always associated with an increase in protein expression. Powley and colleagues have proposed that levels of ERCC1 proteins are highly controlled at the translational level (30). Here, we identified the putative ERCC1-targeting miR-409-3p that behaved differently in ERCC1-positive versus ERCC1-negative tumors (see Fig. 2C).

Concerning the IHC evaluation of ERCC1 expression, the choice of the optimal antibody has been largely discussed in the literature, and no consensus has yet been established. ERCC1 antibodies seem to be particularly sensitive to incubation conditions, and the technical staining procedures have to be optimized for each antibody (31). Furthermore, the ERCC1 epitopes recognized by the different ERCC1 antibodies are unknown. ERCC1 IHC and qRT-PCR discrepancies could therefore be also related to the presence of different ERCC1 isoforms (32). The antibodies used may be specific to one or several ERCC1 isoforms, whereas qRT-PCR primers designed in this study recognized all ERCC1 isoforms.

We further established that ERCC1-negative tumors had a higher rate of genomic aberrations, thus, sustaining a prior hypothesis (9, 28) suggesting that ERCC1 would influence genomic stability with lower number of genomic alterations in DNA in ERCC1-positive tumors. Such a correlation between ERCC1 loss and genomic instability has previously been suggested by a micronuclei study on mice (33). The authors proposed that the increased level of unrepaired lesions and double-strand breaks in ERCC1-deficient mouse fibroblast leads to an increase in mutation frequency and genome instability. As a result of their control function, it is obvious that DNA repair proteins are essential for maintaining the genome integrity (7). However, prior to our analysis, no data were available concerning a direct association between genomic instability and ERCC1 IHC status in NSCLC tumors. Our results, however, should allow a better understanding of the intrinsic prognosis value of ERCC1 protein expression in resected NSCLC.

We also investigated if the presence of mutations was related to ERCC1 expression. TP53 mutations were the most frequently observed (27% of patients). This elevated frequency of TP53 mutations is common in NSCLC, but the influence of TP53 mutations on survival and drug resistance is largely controversial (26, 34–38). Concerning the EGFR gene, many reports have described that EGFR inhibitors (erlotinib, gefitinib, ...) increase survival in NSCLC patients harboring activating EGFR mutations (39). We thus searched for EGFR mutations at a mutational "hot-spot" within the kinase domain of the EGFR gene (exons 18–21; ref. 40). We did not observe increased number of mutations (for EGFR and the other genes) in ERCC1-negative tumors. This is in contrast with previous studies.
which directly linked EGFR mutations with low ERCC1 expression (19, 20). The low number of EGFR mutations found in this study can be explained by the low percentage of never-smokers in this cohort (3%; Table 1). EGFR mutations have indeed been linked to the smoking status of patients (41).

Altogether, our results suggest that ERCC1 influences the rate of gains/losses of large genome segments rather than

**Figure 4.** Differences in gene expression between ERCC1-positive and -negative tumors. A, Heatmap and 2D hierarchical clustering (Ward distance) for transcriptome analysis. B, boxplots repartition of mRNA expression according to ERCC1 IHC status for 7 genes associated with DDR.
the rate of single nucleotide modifications. The underlying molecular mechanisms remain unclear, but ERCC1 is a general structure-specific endonuclease implicated in several important processes assuring DNA integrity, such as single-strand annealing, Fanconi anemia-dependent ICL repair, recombination processes, and telomere protection (42).

Our study also showed that ERCC1-positive tumors do not have a strong distinct pattern of gene expression in key pathways such as HER signaling, phosphoinositide 3-kinase-PTEN-AKT-mTOR signaling or the RAF-RAS-MEK (MEK: mitogen-activated protein/extracellular-signal regulated kinase) pathway. However, we identified 24 genes linked to cell death (GO:0008219) and 3 genes overexpressed in ERCC1-positive tumors that were linked to EGFR signaling pathway ITGA6, KRT16, KRT6B (GO:0007173). Interestingly, ERCC1-positive tumors seemed to share a common DDR phenotype leading to the overexpression of 7 genes strongly linked to DDR (GO: 0006974).

We further identified a 29 miRNA signature in which there was one remarkable miRNA, miR-375, which was downregulated in ERCC1-positive patients. Interestingly, miR-375 was shown to be downregulated in gastric and hepatocellular cancer (HCC) tissues compared with adjacent normal tissues (43, 44). Furthermore, miR-375 inhibited proliferation and invasion of HCC cells (44). Recent reports have suggested that miR-375 may function as a tumor suppressor by regulating cell proliferation through targeting of the Janus-activated kinase 2 oncogene (43). Moreover, it has been suggested that 3'-phosphoinositide-dependent protein kinase 1), a key component in the Akt signaling pathway that regulates cancer cell survival and proliferation, is another direct target of miR-375 (45). Altogether, we cannot exclude that miR-375 downregulation in ERCC1-positive tumors induces a proliferative advantage in these tumors. With a target prediction program (miRGen), we identified, among the genes predicted to be targeted by miR-375, 9 genes linked to DNA repair: TP53, USP1, APEX1, TYMS, MLH3, XRCC6BP1, ERCC3, NTHL1, and PARP4.

Thus, downregulation of this miRNA could also contribute to the increased DDR phenotype in ERCC1 positive tumors.

In summary, this study aimed to establish the ID-card of ERCC1-negative versus ERCC1-positive tumors in resected NSCLC. Our data suggest that miRNA may play a role in the regulation of ERCC1 protein expression. Remarkably, ERCC1 protein expression was associated with genomic instability in terms of increased genomic aberration rates. Genomic instability has been proposed to be both a cause and a consequence of carcinogenesis. Therefore, an association between ERCC1 and genomic instability can provide new insight into the pathogenesis of NSCLC tumors, particularly in our effort to explain the intrinsic prognostic impact of ERCC1 in untreated patients. ERCC1 status alone did not have a significant prognostic or predictive value in this small cohort. There is an increasing evidence that combination of biomarkers representing multiple pathways of DNA repair increases the predictive power of these biomarkers. DNA repair is a multiplayer multidimensional process, which evaluation should not be restricted to ERCC1. In fact, a multiparametric evaluation of different key players of DNA repair (NER/ERCC1, BER/PARP, HR/BRCa, etc) should be encouraged along with functional tests of DNA repair capacity or global readouts of DNA repair dysfunctionality (i.e., rate of aberrant genome).

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Grant Support

This work was supported by grants from CHEMÔRES EU FP6 and Institut National du Cancer INCa "projet libre."

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received March 24, 2011; revised May 19, 2011; accepted June 20, 2011; published OnlineFirst July 12, 2011.

References

Molecular Characteristics of ERCC1-Negative versus ERCC1-Positive Tumors in Resected NSCLC

Luc Friboulet, Daniel Barrios-Gonzales, Frédéric Commo, et al.


Updated version
Access the most recent version of this article at:
doi:10.1158/1078-0432.CCR-11-0790

Supplementary Material
Access the most recent supplemental material at:
http://clincancerres.aacrjournals.org/content/suppl/2011/07/12/1078-0432.CCR-11-0790.DC1

Cited articles
This article cites 45 articles, 16 of which you can access for free at:
http://clincancerres.aacrjournals.org/content/17/17/5562.full.html#ref-list-1

Citing articles
This article has been cited by 8 HighWire-hosted articles. Access the articles at:
/content/17/17/5562.full.html#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.