Epigenetic Inactivation of the Chromosomal Stability Control Genes \textit{BRCA1}, \textit{BRCA2}, and \textit{XRCC5} in Non–Small Cell Lung Cancer

Ming-Ni Lee,¹ Ruo-Chia Tseng,¹ Han-Shui Hsu,² Jia-Yang Chen,¹ Ching Tzao,³ William L. Ho,⁴ and Yi-Ching Wang¹,⁵

Abstract

Purpose: Lung cancer cells frequently exhibit marked chromosome instability. We postulated that alterations of the double-strand break repair genes (\textit{BRCA1}, \textit{BRCA2}, and \textit{XRCC5}) might be involved in lung cancer.

Patients and Methods: We examined the loss of protein and mRNA expression and the 5’CpG hypermethylation and allelic imbalance of the \textit{BRCA1}, \textit{BRCA2}, and \textit{XRCC5} genes in 98 non–small cell lung cancer (NSCLC) samples. Anchorage-dependent growth after reexpression of these genes was examined in a lung cancer cell line that originally lacked \textit{BRCA1} and \textit{BRCA2} expression.

Results: The data indicated that low protein expression of \textit{BRCA1} and \textit{BRCA2} was frequent in lung adenocarcinomas (42–44%), whereas low \textit{XRCC5} protein expression was more prevalent among squamous cell carcinoma (32%). In addition, low \textit{BRCA1} expression was significantly associated with low RB expression, especially in lung adenocarcinoma. Concurrent alterations in \textit{XRCC5} and \textit{p53} were the most frequent profiles in smoking patients. Importantly, low mRNA and protein expressions of \textit{BRCA1}, \textit{BRCA2}, and \textit{XRCC5} were significantly associated with their promoter hypermethylation. 5-Aza-2’-deoxycytidine treatment of NSCLC cells showed demethylation and reexpression of the \textit{BRCA1} and \textit{BRCA2} genes and reduced anchorage-independent growth.

Conclusions: Our retrospective study provides compelling evidence that low mRNA and protein expression in the \textit{BRCA1}/\textit{BRCA2} and \textit{XRCC5} genes occur in lung adenocarcinoma and squamous cell carcinoma, respectively, and that promoter hypermethylation is the predominant mechanism in deregulation of these genes. Alteration of the double-strand break repair pathway, perhaps by interacting with \textit{p53} and RB deregulation, is important in the pathogenesis of a subset of NSCLC.

Lung cancer is one of the most common malignancies, making it very important to identify the associated gene(s) involved. We previously did a genome-wide loss of heterozygosity (LOH) study in 71 primary surgically resected non–small cell lung cancer (NSCLC) tumors in which a high mean LOH frequency was reported, indicating the presence of chromosome instability in NSCLC (1). Chromosome instability may be caused by failure in the repair of DNA double-strand breaks (DSB; ref. 2). Interestingly, the genome regions at 2q33-35 and 13q12.3, which included gene loci encoding the \textit{XRCC5} (\textit{Ku80}) and \textit{BRCA2} (breast cancer 2) DSB repair proteins, respectively, showed a high frequency of LOH in NSCLC tumors (1). We therefore postulated that alterations of genes in chromosomal stability control pathways, such as DSB repair genes (\textit{BRCA1}, \textit{BRCA2}, and \textit{XRCC5}), may be involved in NSCLC tumorigenesis.

Two distinct and complementary mechanisms, homologous recombination and nonhomologous end joining, are involved in the repair of detrimental DSBs in mammalian cells (2, 3). The breast tumor suppressors \textit{BRCA1} and \textit{BRCA2} have been shown to interact with DNA damage–induced Rad51 protein to form foci on DSBs (4, 5). Mouse \textit{Brca1} and \textit{Brca2}-deficient embryonic stem cells show a reduced efficiency of homologous recombination (6, 7) and develop spontaneous chromosomal aberrations (8, 9). It has been shown that \textit{XRCC5} and \textit{XRCC6} encode the 80- and 70-kDa subunits of the Ku70/Ku80 heterodimer, which may be involved in the binding of DSB ends during nonhomologous end joining (10). Primary fibroblasts from \textit{Xrcc5}−/− mice were found to frequently acquire chromosomal aberrations (11). Growing evidence...
suggested the potential involvement of defects in the DSB repair system in the chromosome instability of human cancers.

Germ line mutations in the BRCA1 and BRCA2 genes are strongly associated with an elevated risk of breast and ovarian cancers (12). Mutation carriers have a statistically increased risk of several other cancers [i.e., pancreatic and cervical cancers with BRCA1 (13) and prostate, pancreas, stomach, melanoma, lung, and bladder cancers with BRCA2 (14)]. Decreased protein expression of XRCC5 is also reported for melanoma, cervical, and colon cancers (15). Frequent LOH at the BRCA1, BRCA2, and XRCC5 has been observed in several sporadic cancers other than breast and ovary (i.e., tumors of the colon, pancreas, soft tissue, and lung; refs. 1, 16). Recently, promoter hypermethylation of the BRCA1 and BRCA2 has been shown in sporadic breast and ovarian tumors (17, 18). However, no direct evidence regarding the potential involvement of defects in the DSB repair system has yet been obtained in lung cancer. In the data to date, only two studies have reported the mRNA expression (19) and 5’CpG methylation (20) analyses on the BRCA1 gene in lung cancer. No XRCC5 studies have been reported previously in lung cancer.

To investigate the clinical association of DSB repair alteration in NSCLC tumorigenesis and to elucidate the possible mechanisms involved, we did gene/protein alteration studies, including LOH and promoter hypermethylation, and loss of RNA and protein expression on the DSB repair genes (BRCA1, BRCA2, and XRCC5) in tumors from 98 NSCLC patients. The clinicopathologic data of patients and anchorage-independent growth in model cells were also examined to determine the associations of DSB repair alteration with the clinical and biological behavior of NSCLC tumorigenesis.

Materials and Methods

Sample preparation and clinical characterization of patients. Tissues were collected after obtaining appropriate institutional review board permission and informed consent from the recruited patients. Surgically resected tumor samples from 98 patients with NSCLC were collected between 1999 and 2004. Of these patients, 52 had adenocarcinomas, 37 had squamous cell carcinomas (SCC), 6 had large cell carcinomas, and 3 had nondiagnostic NSCLC. The histology of the tumor types and their stages were determined according to the WHO classification and the tumor-node-metastasis system, respectively. Information on the age, sex, and smoking history of the patients was obtained from hospital records.

Surgically resected tumor samples were immediately snap-frozen and subsequently stored in liquid nitrogen. For the LOH and methylation assay, genomic DNA from matched pairs of primary tumors and nearby normal lung tissues was prepared using proteinase K digestion and then ethanol precipitation. For the normal lung tissues was prepared using proteinase K digestion and genomic DNA from matched pairs of primary tumors and nearby normal tissues, using Trizol reagent (Invitrogen, Carlsbad, CA). cDNA was phenol-chloroform extraction and then ethanol precipitation. For the LOH and methylation records.

The tumor-node-metastasis system, respectively. Information on the age, sex, and smoking history of the patients was obtained from hospital records.

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LOH analysis. PCR reactions were conducted using fluorescently labeled and unlabeled primers for the microsatellite marker(s) located in the chromosome instability of human cancers.

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5-Aza-2’-deoxycytidine treatment of NSCLC cells. The human NSCLC cell line CL1-5-F4 was derived from Taiwanese patients with
lung adenocarcinoma (kindly provided by Dr. P-C. Yang, Department of Internal Medicine, National Taiwan University Hospital, Taipei, Taiwan). Cells were plated at 10^5 per 100-mm culture dish on the day before treatment. The cultures were treated for two to three doubling times with 1 µmol/L of 5-aza-2'-deoxycytidine (5-Aza-dC). The 5-Aza-dC–containing medium was changed for each cell doubling during the treatment. On the day after the third doubling, the cells were harvested for an analysis of their methylation status using a MSP assay and of their production of mRNA using a RT-PCR assay.

**Anchorage-independent growth assay.** Cells (1 × 10^4) were seeded in the upper layer with 0.4% agarose gel, 1 µmol/L of 5-Aza-dC, and 10% serum containing medium. The lower layer contained 0.6% agarose gel and 1 µmol/L of 5-Aza-dC. Cells were fed with medium containing 1 µmol/L of 5-Aza-dC and 10% serum for 10 days. After 10 days of treatment, the cell mass growth over 50 μm was counted.

**Statistical analysis.** Pearson’s χ² test was used to compare the frequency of BRCA1, BRCA2, and XRCC5 alterations in NSCLC patients with different characteristics, including age, sex, and smoking status, and various clinicopathologic variables, such as tumor type and tumor stage. The smokers included both current smokers and ex smokers. The comparison of tumor type focused mainly on adenocarcinoma versus SCC. For the staging of the primary tumor, we pooled stages I and II for the early-stage category and stages III and IV for the late-stage category.

**Results**

**Gene and protein alterations of BRCA1, BRCA2, and XRCC5 in primary NSCLC tumors**

Loss of protein expression and its correlation with clinicopathologic variables and p53/RB alterations. Immunohistochemical staining of BRCA1, BRCA2, and XRCC5 proteins was done on 98 tumor samples. The data indicated that 37%, 34%, and 21% of tumors showed an absence or low expression of BRCA1, BRCA2, and XRCC5 proteins, respectively (Fig. 1B, D, and H; Table 1). In addition, we found that low expression of either BRCA1 or BRCA2 proteins was in 57% of tumors analyzed (Table 1).

To examine whether there were associations of alterations at the BRCA1, BRCA2, and XRCC5 proteins with the clinical characteristics of patients, the occurrence of alteration at each protein was compared with the patients’ clinicopathologic variables, including sex, age, smoking habit, tumor type, and tumor stage (Table 1). χ² analysis showed that no significant correlation of BRCA1, BRCA2, and XRCC5 protein expressions was detected in these clinicopathologic variables. However, a significant correlation of BRCA1 or BRCA2 protein expression was found with the tumor type, with adenocarcinoma being more likely to have low expression of either protein compared to SCC (Table 1).

**Table 1.** Correlation analyses of BRCA1/BRCA2/XRCC5 protein alteration in relation to clinicopathologic variables of 98 NSCLC patients

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>BRCA1</th>
<th></th>
<th>BRCA2</th>
<th></th>
<th>BRCA1 or BRCA2</th>
<th></th>
<th>XRCC5</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total</td>
<td>Low protein, n (%)</td>
<td>Total</td>
<td>Low protein, n (%)</td>
<td>Total</td>
<td>Low protein, n (%)</td>
<td>Total</td>
<td>Low protein, n (%)</td>
</tr>
<tr>
<td>Overall</td>
<td>98</td>
<td>36 (37)</td>
<td>98</td>
<td>33 (34)</td>
<td>98</td>
<td>56 (57)</td>
<td>98</td>
<td>21 (21)</td>
</tr>
<tr>
<td>Smoking</td>
<td></td>
<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>56</td>
<td>21 (38)</td>
<td>56</td>
<td>19 (34)</td>
<td>56</td>
<td>32 (57)</td>
<td>56</td>
<td>13 (23)</td>
</tr>
<tr>
<td>No</td>
<td>30</td>
<td>10 (33)</td>
<td>30</td>
<td>13 (43)</td>
<td>30</td>
<td>18 (60)</td>
<td>30</td>
<td>3 (10)</td>
</tr>
<tr>
<td>Tumor type</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Adenocarcinoma</td>
<td>52</td>
<td>22 (42)</td>
<td>52</td>
<td>23 (44) 0.054</td>
<td>52</td>
<td>36 (69) 0.014</td>
<td>52</td>
<td>8 (15)</td>
</tr>
<tr>
<td>SCC</td>
<td>37</td>
<td>11 (30)</td>
<td>37</td>
<td>9 (24)</td>
<td>37</td>
<td>16 (43)</td>
<td>37</td>
<td>12 (32) 0.058</td>
</tr>
<tr>
<td>Tumor stage</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I + II</td>
<td>50</td>
<td>16 (32)</td>
<td>50</td>
<td>16 (32)</td>
<td>50</td>
<td>27 (54)</td>
<td>50</td>
<td>13 (26)</td>
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<tr>
<td>III + IV</td>
<td>44</td>
<td>19 (43)</td>
<td>44</td>
<td>17 (39)</td>
<td>44</td>
<td>28 (64)</td>
<td>44</td>
<td>7 (16)</td>
</tr>
</tbody>
</table>

NOTE: χ² with significance are shown as superscripts.

*Total number of samples in some categories is less than the overall number analyzed because clinical data was not available for these samples.
low BRCA1 or BRCA2 protein expression occurred primarily in patients suffering from adenocarcinoma types of lung cancer (P = 0.014). By contrast, low protein expression of the XRCC5 was nearly significantly restricted to patients suffering from SCC of lung cancer (P = 0.058).

In this cohort of patients, 81 patients had been analyzed for the protein expression of p53 and RB in our lab previously (29, 30). Therefore, the alterations of BRCA1, BRCA2, and XRCC5 proteins were tested for their association with the alterations in p53 and RB to reveal the existence of a correlation of DSB repair with p53/RB pathway (Table 2). The data indicated that a loss of both BRCA1 and RB protein expression was more in adenocarcinoma patients than that in SCC patients suffering from adenocarcinoma types of lung cancer (P = 0.058).

Table 2. Correlation analyses of low protein expression of BRCA1/BRCA2/XRCC5 and RB/p53 protein alteration in relation to tumor type and smoking habit

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Total, n*</th>
<th>BRCA1, low protein, n (%)</th>
<th>BRCA2, low protein, n (%)</th>
<th>XRCC5, low protein, n (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein expression of RB in AD patients</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low</td>
<td>22</td>
<td>14 (64) 0.020</td>
<td>9 (41)</td>
<td>5 (23)</td>
</tr>
<tr>
<td>Normal</td>
<td>13</td>
<td>3 (23)</td>
<td>6 (46)</td>
<td>1 (8)</td>
</tr>
<tr>
<td>Protein expression of p53 in smoker patients</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Overexpression</td>
<td>27</td>
<td>9 (33)</td>
<td>9 (33)</td>
<td>9 (33) 0.084</td>
</tr>
<tr>
<td>Normal</td>
<td>29</td>
<td>12 (41)</td>
<td>10 (34)</td>
<td>4 (14)</td>
</tr>
</tbody>
</table>

NOTE: P s with significance are shown as superscripts.

*Although a total of 81 patients were analyzed, only the data for 35 adenocarcinoma patients and 56 smoker patients were shown.

Concordance analysis of low mRNA/protein expression of BRCA1, BRCA2, and XRCC5 with their promoter hypermethylation and allelic imbalance

To investigate whether the low BRCA1, BRCA2, and XRCC5 mRNA and proteins expressions were coordinated with promoter hypermethylation and allelic imbalance at the gene locus, cross-tabulation analysis was done on the protein data to examine the correlations among mRNA expression, DNA methylation, and LOH status, using the Pearson’s χ² test (Fig. 5). Aberrant protein expression was significantly associated with low mRNA expression (BRCA1, P < 0.001; BRCA2, P < 0.001; XRCC5, P = 0.032) and with promoter hypermethylation (BRCA1, P = 0.008; BRCA2, P < 0.001; XRCC5, P = 0.047) but not with LOH of the gene. Furthermore, low mRNA expression was significantly associated with hypermethylation of the gene (BRCA1, P < 0.001; BRCA2, P < 0.001; XRCC5, P < 0.001).

LOH at microsatellites in the three gene loci. We further investigated whether LOH of the BRCA1, BRCA2, and XRCC5 genes occurred in this series of NSCLC tumors (Fig. 4). Microdissected genomic DNA obtained from 87 matched pairs of primary tumors and nearby normal lung tissue was examined for the incidence of LOH at the intragenic and/or nearby microsatellite markers. The data indicated that 25%, 44%, and 48% of tumors were found to harbor LOH at the BRCA1, BRCA2, and XRCC5 loci, respectively.

Fig. 2. Representative figures for BRCA1, BRCA2, and XRCC5 mRNA expression analysis by the semiquantitative multiplex RT-PCR assay. The results of their mRNA expression status are indicated above. + (positive) and − (negative) in the rows labeled mRNA indicate their expression status. N, normal lung tissue; T, tumor tissue of the lung. β-actin was used as the internal control for the analysis (an amplification fragment of 201 bp was used for BRCA1 and BRCA2 analyses; an amplification of 618 bp was used for XRCC5 analysis). The tumor tissues expressing low amounts of target transcript are indicated with arrows.
Reactivation of BRCA1 and BRCA2 by 5-Aza-dC treatment

5-Aza-dC increased mRNA expression. To determine whether BRCA1 and BRCA2 promoter methylation could be further linked to the loss of gene expression, CL1-5F4 NSCLC cell, which showed negative expression and promoter hypermethylation of the BRCA1 and BRCA2 genes, were treated with the demethylating agent 5-Aza-dC. As shown in the Fig. 3C, 5-Aza-dC successfully restored mRNA and protein expression and demethylated the promoter region in the cells that lacked BRCA1 and BRCA2 expression and harbored a methylated respective promoter.

5-Aza-dC decreased anchorage independence. To evaluate whether the reexpression of BRCA1 and BRCA2 resulted in low tumorigenicity, the effect of 5-Aza-dC on CL1-5F4 cancer cell growth was tested by the anchorage-independent assay. A significant decrease of anchorage-independent growth was observed in CL1-5F4 cancer cells reexpressing the BRCA1 and BRCA2 (Fig. 3D).

Discussion

Previous studies have shown that lung cancer cells frequently exhibit marked chromosome instability (1, 31). It is possible that defects in the DSB repair system are involved in the genesis of chromosome instability in lung cancer. In an effort to better understand the molecular pathogenesis of the DSB repair pathway in NSCLC, we collected 98 primary NSCLC tumors and carried out a comprehensive molecular analysis, including mRNA/protein expression and DNA methylation/allelic imbalance of three genes (BRCA1, BRCA2, and XRCC5) encoding proteins known to function in the DSB repair pathway. We found that 64% (63 of 98) of NSCLC tumors showed alteration in one of the abovementioned genes, suggesting that DSB repair is potentially involved in the NSCLC tumorigenesis. Low expression of BRCA1 and XRCC5 was frequent in adenocarcinoma/RB−/deficient and SCC/p53−/deficient lung cancer, respectively. Note that low mRNA and protein expression of the BRCA1, BRCA2, and XRCC5 genes was most often attributable to their promoter hypermethylation. To our knowledge, our study is the first to identify deregulation of BRCA1, BRCA2, and XRCC5 genes in the same series of NSCLC samples. Our data provide new evidence for an alternative mechanism of NSCLC tumorigenesis by DSB repair deregulation.

Low levels of BRCA1/BRCA2 and XRCC5 protein expression occurred specifically in primary adenocarcinoma and SCC lung tumors, respectively. It is possible that the homologous recombination and nonhomologous end joining repair pathways differ in their requirements for DSB repair in different cell types (32). Homologous recombination may be responsible for the homologous template in non–smoking-related adenocarcinoma lung cancer development, whereas nonhomologous end joining may be required in the double-stranded end joining for smoking-related SCC lung cancer. In view of the apparent link between RB and p53 tumor suppressor genes in adenocarcinoma and smoker NSCLC tumorigenesis, respectively (29, 33), in this series of NSCLC patients with previously examined protein expression data of the RB and p53, we investigated the possible relationship of either BRCA1/BRCA2 or XRCC5 alterations. The data indicated that low BRCA1 expression was significantly associated with low RB expression, especially in adenocarcinoma-type lung cancer (Table 2). In addition, concurrent alterations in XRCC5 and p53 were the most frequent profile in smoking NSCLC patients (Table 2). These data were in agreement with the role of p53 and RB in the cell cycle checkpoint controls (34, 35). It has been shown that p53 deficiency can rescue the embryonic lethality and developmental defects of XRCC5−/− mice (11). RB binds to the BRCT domain of BRCA1 to mediate the ability of BRCA1 to suppress growth (36, 37). We therefore hypothesized a model in which alteration of the DSB repair pathway, perhaps by interacting with p53 and RB deregulation, is important in the
pathogenesis of a subset of NSCLC. The function of these "gatekeepers" (p53 and Rb) and "caretakers" (XRCC5 and BRCA1/BRCA2) in regulating the cellular response to DSB in NSCLC warrants further investigation.

A high concordance was observed between alterations in protein and mRNA expression and promoter hypermethylation of the BRCA1, BRCA2, and XRCC5 genes. The data suggest that promoter hypermethylation is the predominant mechanism to inactivate the BRCA1, BRCA2, and XRCC5 genes. We deliberately designed the primers for MSP at regions with reported promoter activities (38–41). Methylation status of the promoter region examined was correlated to the transcriptional activity of the genes and perhaps to tumorigenesis. This conclusion is further strengthened by the reexpression of mRNA expression together with demethylation at the promoter region in both the BRCA1 and BRCA2 genes and inhibition of anchorage-independent growth, using 5-Aza-dC treatment for NSCLC cell lines (Fig. 3C and D). In the future, it will be important to examine the effects of 5-Aza-dC on neoplastic phenotypes within a much larger cohort of samples so as to define more precisely the critical site in BRCA1, BRCA2, and XRCC5 for gene silencing.

Note that some patients with positive protein expression showed promoter methylation. This could be explained by the presence of several distinct tumor subpopulations, one of which has methylation and does not express the protein, whereas the others have no methylation and no protein expression. In addition, methylation occurred at only one allele so that the remaining allele still expresses the protein that we observed. We found that the allelic imbalance in chromosome regions that harbor the BRCA1, BRCA2, and XRCC5 genes was not associated with the altered expression of the respective proteins. This may be because inactivation of tumor suppressor genes occurs via a two-hit mechanism. Loss of only one allele may not result in loss of tumor suppressor protein expression (42). There were several patients with no demonstrable protein staining and no evidence of promoter methylation and LOH. We postulated that histone deacetylation further decreasing the access of transcription factors to their binding sites on gene promoters and resulting in gene silencing may be involved in these patients. Alternatively, unidentified genetic variants may be occurred in these samples.

In conclusion, our findings provided compelling evidence of low mRNA and protein expression in the BRCA1/BRCA2 and XRCC5 genes in lung adenocarcinoma and SCC, respectively, and suggested that promoter hypermethylation is the predominant mechanism in the deregulation of these genes. Our data support the premise that BRCA1, BRCA2, and XRCC5 are tumor suppressors in NSCLC, and that alteration in DSB repair pathway is involved in NSCLC tumorigenesis. Our clinical correlation data corroborated the cell model data by Sun et al. showing the deficiency of DSB repair in tumorigenic human bronchial epithelial cells treated with α-particle exposure (43). Whether the deficiency of DSB repair could promote tumorigenesis in NSCLC cells treated with other DNA damage reagents, possibly by interacting with p53 and RB deregulation, needs further examination. In addition, BRCA1 has been shown to associate with the RAD50/MRE11/NBS1 complex that functions in both nonhomologous end joining and homologous recombination (44). Therefore, we tested the mRNA alteration of NBS1 in 75 NSCLC patients. The data indicated that 17% of patients had low mRNA expression of NBS1. However, low expression of NBS1 did not show any statistically significant association with clinicopathologic variables. Alterations in other components of DSB signaling, including RAD50, MRE11, XRCC4, and XRCC6, in NSCLC samples are under investigation to more clearly determine the etiology of this disease.

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