Ataxia-Telangiectasia and RAD3-Related and Ataxia-Telangiectasia-Mutated Proteins in Epithelial Ovarian Carcinoma: Their Expression and Clinical Significance

BANGHYUN LEE1*, HEE JIN LEE2*, HYE-YON CHO3, DONG HOON SUH1, KIDONG KIM1, JAE HONG NO1, HAERYOUNG KIM4 and YONG-BEOM KIM1

Abstract. Background/Aim: The expression patterns of the key DNA damage response-related proteins, ataxia-telangiectasia and tfiih/ner complex atp-dependent 5′-3′ dna helicase subunit rad3 (RAD3)-related (ATR) and ataxia-telangiectasia-mutated (ATM) proteins in ovarian cancer are not well-known. This study aimed to evaluate the expressions of ATR and ATM proteins, and to investigate their clinical significance in epithelial ovarian carcinoma (EOC).

Materials and Methods: The expressions of nuclear/cytoplasmic Ser428-phosphorylated ATR (p-ATR) and Ser1981-phosphorylated ATM (p-ATM) were evaluated by immunohistochemistry in 100 patients with EOC. The clinical significances of p-ATR and p-ATM protein expression were evaluated in terms of tumor progression and survival. Results: Low expression of cytoplasmic p-ATR was significantly associated with advanced stage, serous histology, large residual mass, and high preoperative serum CA125 level. Univariate survival analysis revealed that low expression of cytoplasmic p-ATR protein was significantly associated with poor disease-free survival and poor overall survival. Conclusion: Our study demonstrates that cytoplasmic ATR protein might serve as a prognostic biomarker for patients with EOC.

DNA damage response (DDR) is a complex regulatory network of signaling pathways, including cell-cycle checkpoints, DNA repair, and damage tolerance pathways. DDR is associated with various cellular components and processes. The failure of DDR may cause cells to multiply out of control, leading to cancer. DNA-damaging agents are usually used in cancer therapy, and drugs that target DDR enzymes also provide novel opportunities for cancer therapy (1).

The ataxia-telangiectasia and tfiih/ner complex atp-dependent 5′-3′ dna helicase subunit rad3 (RAD3)-related (ATR) and ataxia-telangiectasia-mutated (ATM) protein kinases are master regulators of DDR. ATR is activated in response to various DNA events, including replication interference, and ultraviolet radiation. ATM is activated by DNA double-strand breaks that result from ionization radiation and other agents, or oxidative stress (2). These proteins are included in the group of phosphatidylinositol-3 kinase (PI3K)-related protein kinases, and regulate downstream pathways by phosphorylating multiple substrates, such as the checkpoint CHK1 and CHK2 protein kinases, p53, and targets at the sites of DNA damage (3).

DNA damage may cause ovarian cancer. For example, epithelial ovarian carcinoma (EOC) may be induced from DNA damage by successive ovulation (4). However, the role of DDR in ovarian cancer (especially related to ATR and ATM) has only been evaluated in a few studies. In response to DNA damage, breast and ovarian cancer susceptibility gene 1 (BRCA1) is phosphorylated at various sites by several kinases (e.g. ATM, CHK2, and ATR), and then plays a central role in DDR. Accordingly,
the phosphorylation of BRCA1 by kinases in response to DNA damage may partially explain the mechanism by which BRCA1 mutations induce breast and ovarian cancer (5). Genistein, an isoflavonoid in soybean, has been reported to induce apoptosis of HO-8910 human ovarian cancer cells through activation of ATR-CHK1 and ATM-CHK2 checkpoint pathways (6). Furthermore, in A2780 ovarian carcinoma cells, the ATM-CHK2 pathway was activated by DSBs induced by camptothecin or ionizing radiation (7). The mRNA levels of BRCT-repeat inhibitor (BRIT1), a proximal factor in the ATR and ATM pathway, were also found to be markedly decreased in serous ovarian cancer compared to benign ovarian tissues (8).

DDR is considered to be a critical process for cancer suppression and therapy. However, the expression patterns of DDR-related proteins in ovarian cancer are still unclear. Therefore, the present study aimed to evaluate the expressions of the key DDR-related proteins, Ser428-phosphorylated ATR (p-ATR) and Ser1981-phosphorylated ATM (p-ATM), and to investigate their clinical significance in EOC.

Materials and Methods

Patients. This study was approved by the Institutional Review Board of Seoul National University Bundang Hospital (B-1401/234-301). Our retrospective cohort included patients who underwent staging operations for ovarian cancer at the Seoul National University Bundang Hospital from May 1, 2003 to December 31, 2009. The inclusion criteria were as follows: histologically-confirmed EOC, for which formalin-fixed paraffin-embedded (FFPE) tissue blocks were available, and administration of platinum-based chemotherapy (cisplatin and carboplatin). Patients who underwent neoadjuvant chemotherapy were excluded from the study.

The medical records, pathology reports, and pathology slides of 100 patients who were eligible for the current study were reviewed. Representative FFPE blocks from each case were selected for tissue microarray (TMA) construction. Clinical response to chemotherapy was evaluated using the revised RECIST guidelines (version 1.1) (9). Recurrence of tumor within 6 months of the end of first-line chemotherapy was considered to indicate platinum-resistance. Disease-free survival (DFS) was defined as the time from surgery to the first observation of recurrence. Overall survival (OS) was defined as the time from surgery to death or to the date the study was completed. A tumor with size larger than 1 cm was considered to be a large residual mass.

TMA construction. Tissue cores measuring 2 mm in diameter were obtained from donor primary EOC blocks and arranged into recipient TMA blocks using a trephine apparatus (Superbiochips Laboratories, Seoul, Korea). One or two cores were taken from each specimen, and normal ovarian tissue was also included in the TMA block to serve as a control.

Immunohistochemistry. Four-micrometer-thick sections were cut from TMA blocks for immunohistochemical analysis. Immunohistochemistry was performed using a Benchmark XT autostainer (Ventana, Tucson, AZ, USA). The following primary antibodies were used: rabbit polyclonal anti-phospho-ATR (Ser428) (CST2853; 1:30 dilution; Cell Signaling Technology, Inc., Danvers, MA, USA) and mouse monoclonal anti-phospho-ATM (Ser1981) (clone 10H11.E12, CST4526; 1:200 dilution; Cell Signaling). Antigen retrieval for both antibodies was performed using EDTA at pH 8.4. UltraView Universal DAB Detection Kit (cat #760-500; Ventana) was used for signal detection.

Interpretation of staining and classification of subgroups for each variable. The immunohistochemical staining results were analyzed independently by two pathologists who were blinded to the clinical characteristics of the patients. The expressions of p-ATR and p-ATM protein were evaluated in both nuclear and cytoplasmic compartments (Figure 1). The percentage of cells stained (0-100%) and staining intensity (category 0-3) were used for H-scoring. A score ranging from 0 to 300 was then calculated by multiplying these two variables. Based on the receiver operating characteristic (ROC) curve for resistance to platinum-based chemotherapy, cytoplasmic p-ATR (cutoff score=17.5) and nuclear p-ATM (cutoff score=225.0) expressions were divided into high and low H-score groups. Cytoplasmic p-ATM protein was not detected by immunohistochemistry. Based on the ROC curve for resistance to platinum-based chemotherapy, a value of 52 years was used to partition the patients into an older and a younger age group. Furthermore, a value of 292 U/ml was used to classify the patients into groups with high and low preoperative serum CA125 levels. The cutoff values were chosen to maximize the sum of sensitivity and specificity, as indicated by the ROC curves.

Statistical analyses. Statistical analyses were performed using IBM SPSS Statistics 21 (IBM Corporation, Inc., Chicago, IL, USA). Data for continuous variables are expressed as the mean±the standard deviation (SD). Data for categorical variables are expressed as numbers and percentages. Continuous variables were analyzed using independent t-tests. Categorical variables were analyzed using the Pearson chi-squared test or Fisher’s exact test (two-sided). To evaluate whether specific variables were independent risk factors for DFS and OS, Cox regression analyses were performed with and without adjustment for confounding factors, such as age, stage and type of ovarian cancer, size of the residual mass, preoperative serum CA125 level, and cytoplasmic ATR expression. A value of p<0.05 was considered statistically significant.

Results

Expression of ATR and ATM. For cytoplasmic p-ATR protein (mean H-score=41.1), 62.0% of the patients had low expression, including 45.0% with negative expression (no staining), and 38.0% had high expression. Nuclear p-ATR protein (mean H-score=5.1) was expressed in 13.0% of the patients and was not expressed in 87.0% of the patients. Nuclear p-ATM protein (mean H-score=177.8) was highly expressed in 45.0% of the patients. In contrast, low expression of nuclear p-ATM protein was seen in 55.0% of the patients, including 5.0% who had negative expression with no staining in the cytoplasm (Figure 1, Tables I and II).
Associations of ATR and ATM expression with clinicopathological characteristics. Cytoplasmic expression of p-ATR protein was significantly decreased in patients with advanced-stage disease, serous-type disease, large residual mass, high preoperative serum CA125 levels, development of recurrence, and death (Table I). However, cytoplasmic expression of p-ATR protein was not significantly associated with age.

The following characteristics were not observed to differ significantly according to the degree of nuclear p-ATR protein expression: age (p=0.228), stage (p=0.685) and type (p=0.114) of ovarian cancer, size of residual mass (p=0.100), preoperative serum CA125 levels (p=0.126), development of recurrence (p=0.708), and death (p=0.767). In addition, age (p=0.136), stage (p=0.822) and type (p=0.936) of ovarian cancer, size of residual mass (p=0.246), preoperative serum

Table I. Association of cytoplasmic expression of p-ATR protein with clinicopathological characteristics.

<table>
<thead>
<tr>
<th>Cytoplasmic p-ATR protein expression</th>
<th>Age (years)</th>
<th>FIGO stage</th>
<th>Histological type</th>
<th>Residual mass</th>
<th>Preoperative serum CA125</th>
<th>Recurrence</th>
<th>Death</th>
</tr>
</thead>
<tbody>
<tr>
<td>Low (n=62), n (%)</td>
<td>34 (54.8)</td>
<td>28 (45.2)</td>
<td>44 (71.0)</td>
<td>34 (54.8)</td>
<td>28 (45.2)</td>
<td>39 (62.9)</td>
<td>28 (45.2)</td>
</tr>
<tr>
<td>High (n=38), n (%)</td>
<td>20 (52.6)</td>
<td>18 (47.4)</td>
<td>12 (31.6)</td>
<td>31 (81.6)</td>
<td>7 (18.4)</td>
<td>12 (31.6)</td>
<td>7 (18.4)</td>
</tr>
</tbody>
</table>

p-ATR, Phosphorylated ataxia-telangiectasia and tfiih/ner complex atp-dependent 5'-3' dna helicase subunit rad3 (RAD3)-related protein; FIGO, International Federation of Gynecology and Obstetrics. *Analysis by Fisher’s exact test.

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Table II. Association between platinum resistance and clinicopathological characteristics.

<table>
<thead>
<tr>
<th>Total (n=100), n (%)</th>
<th>Platinum-sensitive (n=86), n (%)</th>
<th>Platinum-resistant (n=14), n (%)</th>
<th>p-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years), mean ± SD</td>
<td>51.5 ± 12.3</td>
<td>53.1 ± 11.3</td>
<td>0.646</td>
</tr>
<tr>
<td>≤52</td>
<td>54 (54.0)</td>
<td>48 (55.8)</td>
<td>6 (42.9)</td>
</tr>
<tr>
<td>&gt;52</td>
<td>46 (46.0)</td>
<td>38 (44.2)</td>
<td>8 (57.1)</td>
</tr>
<tr>
<td>FIGO stage</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Early (I-II)</td>
<td>41 (41.0)</td>
<td>39 (45.3)</td>
<td>2 (14.3)</td>
</tr>
<tr>
<td>Advanced (III-IV)</td>
<td>59 (59.0)</td>
<td>47 (54.7)</td>
<td>12 (85.7)</td>
</tr>
<tr>
<td>Histological type</td>
<td></td>
<td></td>
<td>0.034*</td>
</tr>
<tr>
<td>Serous</td>
<td>56 (56.0)</td>
<td>52 (60.5)</td>
<td>4 (28.6)</td>
</tr>
<tr>
<td>Non-serous</td>
<td>44 (44.0)</td>
<td>34 (39.5)</td>
<td>10 (71.4)</td>
</tr>
<tr>
<td>Mucinous</td>
<td>12 (12.0)</td>
<td>8 (9.3)</td>
<td>4 (28.6)</td>
</tr>
<tr>
<td>Endometrioid</td>
<td>8 (8.0)</td>
<td>8 (9.3)</td>
<td>0 (0.0)</td>
</tr>
<tr>
<td>Clear cell</td>
<td>13 (13.0)</td>
<td>9 (10.5)</td>
<td>4 (28.6)</td>
</tr>
<tr>
<td>Other</td>
<td>11 (11.0)</td>
<td>9 (10.5)</td>
<td>2 (14.3)</td>
</tr>
<tr>
<td>Residual mass</td>
<td></td>
<td></td>
<td>0.031*</td>
</tr>
<tr>
<td>≤1 cm</td>
<td>65 (65.0)</td>
<td>60 (69.8)</td>
<td>5 (35.7)</td>
</tr>
<tr>
<td>&gt;1 cm</td>
<td>35 (35.0)</td>
<td>26 (30.2)</td>
<td>9 (64.3)</td>
</tr>
<tr>
<td>Preoperative serum CA125</td>
<td></td>
<td></td>
<td>0.068</td>
</tr>
<tr>
<td>≤292 U/ml</td>
<td>58 (58.0)</td>
<td>53 (61.6)</td>
<td>5 (35.7)</td>
</tr>
<tr>
<td>&gt;292 U/ml</td>
<td>42 (42.0)</td>
<td>33 (38.4)</td>
<td>9 (64.3)</td>
</tr>
<tr>
<td>Cytoplasmic p-ATR protein expression</td>
<td>High</td>
<td>38 (38.0)</td>
<td>33 (38.4)</td>
</tr>
<tr>
<td>Low</td>
<td>62 (62.0)</td>
<td>53 (61.6)</td>
<td>9 (64.3)</td>
</tr>
<tr>
<td>Nuclear p-ATR protein expression</td>
<td>Yes</td>
<td>13 (13.0)</td>
<td>10 (11.6)</td>
</tr>
<tr>
<td>No</td>
<td>87 (87.0)</td>
<td>76 (88.4)</td>
<td>11 (78.6)</td>
</tr>
<tr>
<td>Nuclear p-ATM protein expression</td>
<td>High</td>
<td>45 (45.0)</td>
<td>37 (43.0)</td>
</tr>
<tr>
<td>Low</td>
<td>55 (55.0)</td>
<td>49 (57.0)</td>
<td>6 (42.9)</td>
</tr>
</tbody>
</table>

p-ATR, Phosphorylated ataxia-telangiectasia and tfiih/ner complex atp-dependent 5'-3' dna helicase subunit rad3 (RAD3)-related protein; p-ATM, phosphorylated ataxia-telangiectasia-mutated protein; FIGO, International Federation of Gynecology and Obstetrics. *Recurrence of tumor within 6 months after the end of first-line chemotherapy was considered to indicate platinum-resistance. *Analysis by Fisher’s exact test.
p-ATM protein expression were not significantly associated with resistance to platinum-based chemotherapy (Table II).

Survival rates. In the total study population, the median DFS was 28 months (range=1-117 months). Univariate analyses indicated that older age, advanced-stage cancer, serous-type disease, large residual mass, high preoperative serum CA125 level, and low expression of cytoplasmic p-ATR protein were significantly associated with an increased risk of recurrence and poorer DFS ($p<0.05$; low cytoplasmic p-ATR protein expression: hazard ratio (HR)=2.2, 95% confidence interval (CI)=1.2 to 4.2). In a multivariate analysis that was adjusted for confounding factors, advanced-stage cancer remained significantly associated with an increased risk of recurrence and poorer DFS (HR=6.5, 95% CI=2.5 to 16.8, $p<0.001$; Table III and Figure 2).

In the total study population, the median OS was 62.5 months (range=1-117 months). Univariate analyses indicated that advanced-stage cancer, large residual mass, high preoperative serum CA125 level, and low cytoplasmic expression of p-ATR protein were significantly associated with an increased risk of death and poorer OS ($p<0.05$; low cytoplasmic p-ATR protein expression: HR=2.3, 95% CI=1.2 to 6.1). In a multivariate analysis that was adjusted for confounding factors, advanced-stage cancer remained significantly associated with an increased risk of death and poorer OS (HR=8.9, 95% CI=2.6-30.0, $p<0.001$; Table IV and Figure 2).

Discussion

Because EOC-related morbidity and mortality remain unacceptable, there exists an outstanding need for targeted-therapies, in addition to conventional chemotherapy. The ATR and ATM pathways are activated in response to the inhibition

Figure 1. Immunohistochemical staining results. A: Diffuse strong nucleocytoplasmic expression of phosphorylated ataxia-telangiectasia and tfih/ner complex ATP-dependent 5’-3’ DNA helicase subunit rad3 (RAD3)-related (p-ATR) is seen in the tumor cells, while the adjacent stroma is negative. B: Weak nuclear p-ATR is seen in this case, without cytoplasmic p-ATR expression. C: Diffuse strong nuclear expression of phosphorylated ataxia-telangiectasia-mutated (p-ATM) is seen in the tumor cells. D: p-ATM is expressed in occasional nuclei in this case. Original magnification, x400.
of replication, such as is prompted by chemotherapy. These normal ATR and ATM pathways can serve to increase cancer cell survival, suggesting a mechanism of resistance to chemotherapy. Therefore, targeting the ATR and ATM pathways is considered to constitute a promising therapeutic method. Many studies have demonstrated that tumor cells can be sensitized to drugs by inhibiting the ATR and ATM pathways in combination with the administration of various chemotherapeutic agents (10-12). ATR and ATM inhibitors have also been shown to radiosensitize tumor cells (13). Therefore, targeting ATR and ATM while administering chemotherapy or radiotherapy is considered to be an attractive therapeutic method. It has been reported that the use of siRNA to inhibit ATR sensitizes HeLa cells to platinum compounds such as cisplatin, carboplatin, and oxaliplatin (14). However, another study found that targeted functional inhibition of ATM in p53-deficient cells did not increase sensitivity to platinum compounds (15). Therefore, we expected that the expression of ATR in EOC would at least be higher in the platinum-resistant group than in the platinum-sensitive group. However, our results showed that p-ATR and p-ATM expressions were not dependent on platinum agent use, suggesting that the regulation of platinum resistance occurs through processes other than the ATR and ATM pathways in EOC (Table II).

Several types of cancers induce a typical stress response involving an acute DDR that is followed by a chronic response in which selected DNA-repair pathways are suppressed in coordination (such as the ATM, CHK1, CHK2, and p53 pathways). However, the exact mechanisms of this response remain unclear (16, 17). It might also be possible for these types of cancer to show an altered response to conventional genotoxic therapies because the signaling pathways that are responsive to DNA damage might be suppressed (17). Therefore, at the start of the current study, we presumed that ATR and ATM proteins would exhibit low expression in advanced EOC and, furthermore, that low expression of ATR and ATM would be associated with chemotherapy resistance; the patterns of DDR would differ in specific cell types, depending on specific conditions. A study of gastric cancer recently showed that ATM, CHK1, and p53 losses were significantly associated with advanced stage and poor DFS, suggesting that they have roles in the progression of gastric cancer (18). Interestingly, in our study, low expression of cytoplasmic p-ATR was significantly associated with advanced-stage disease, as well as increased risks of recurrence and death (and poor DFS and OS; Table I, III and IV, and Figure 2). However, low expression of p-ATM was not significantly associated with these outcomes. The partial or complete loss of ATM-CHK2 signaling is associated with poorer survival for cells and organisms, although cancer may frequently develop because of genomic instability (partially) and oncogenic

Figure 2. Survival analysis according to cytoplasmic expression of phosphorylated ataxia-telangiectasia and tfih/ner complex atp-dependent 5'-3' dna helicase subunit rad3 (RAD3)-related protein. A: Disease-free survival (DFS). B: Overall survival (OS). Univariate Cox regression analyses were performed.
mutations (17). However, ATR-CHK1 signaling has been shown to be essential for the proliferation and survival of many cell types, presumably because partial or complete inactivation of ATR may lead to cell death (such as apoptosis) rather than to oncogenic mutations. This observation suggests that ATR is a promising target for anticancer chemotherapy (17, 19, 20). However, even though they develop rarely, somatic mutations of ATR-CHK1 signaling have been reported in sporadic stomach, endometrial, and colorectal tumors with microsatellite instability (17, 21, 22). It has been suggested that the inhibition of ATR-CHK1 expression in carcinomas that have a mismatch repair deficiency induces chromosome instability, leading to genomic instability and tumorigenesis (22). In our study, low expression of cytoplasmic p-ATR was significantly associated with advanced stage of EOC, suggesting that inhibition of ATR may play a role as a risk factor in cancer without mismatch repair deficiency (Tables I, III, and IV; Figure 2). Further studies are needed to clarify cytoplasmic DNA damage (e.g. mitochondrial DNA) related to cytoplasmic ATR in EOC.

ATR protein that is expressed in the nucleus has antiapoptotic functions and also functions in apoptotic pathways in DDR (19, 20, 23). Recently, the function of cytoplasmic ATR as an antiapoptotic protein has been reported. Cytoplasmic ATR had a checkpoint kinase-independent role in inhibiting the B-cell leukemia/lymphoma 2 (BCL2) associated X (BAX)-mediated apoptotic pathways in mitochondria following UV irradiation (20). In addition, ATM protein is predominant in the nucleus. Moreover, the presence of ATM protein has also been documented in the cytoplasm of some cell types. Cytoplasmic functions of ATM in the activation of PI3K- v-akt murine thymoma viral oncogene homolog 1 (AKT) have also been discovered, suggesting the potential for cytoplasmic ATM protein kinase as a therapeutic target of cancer (24). In addition, differential localization of ATM in the cytoplasm and nucleus is reportedly correlated with activation of distinct downstream signaling pathways (25). Therefore, we investigated the expressions of ATR and ATM proteins in cytoplasmic and nuclear compartments of EOC. In our study, low cytoplasmic p-ATR protein expression was dominant (compared to high expression) and cytoplasmic p-ATM protein expression was not detected. Low cytoplasmic p-ATR protein expression was also significantly associated with aggressive clinicopathological characteristics, including advanced stage of EOC and
poorer survival (Tables I, III, and IV; Figure 2). These findings demonstrate that ATR expression is negatively correlated with the severity of ovarian cancer. It has been reported that nuclear proteins [such as epithelial Cell Transforming Sequence 2 (Ect2)] increase in advanced EOC and play a role in its transformation, even though cytoplasmic proteins do not (26). However, our study of EOC showed that the incidence of nuclear p-ATR protein expression was very low, and that the incidence of nuclear p-ATM protein expression was similar in both the high and low expression groups (Table II). Expression patterns of nuclear p-ATR and p-ATM proteins were also not associated with the severity of clinicopathological characteristics in cases of EOC. These findings suggest that nuclear ATR and ATM proteins do not play critical roles in the progression of EOC.

In conclusion, this study demonstrates that ATR and ATM pathways are not associated with the occurrence of platinum resistance. Our results also demonstrate that low expression of cytoplasmic ATR protein may have potential as a prognostic factor of EOC. Indeed, our findings suggest that cytoplasmic ATR protein is negatively correlated with prognosis of EOC. Overall, our results significantly improve the understanding over the role of DDR in ovarian cancer, especially for ATR and ATM pathways.

Acknowledgements

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Conflicts of Interest

The Authors declare that they have no conflict of interest with regard to this study.

References


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