Abnormal expression of p-ATM/CHK2 in nasal extranodal NK/T cell lymphoma, nasal type, is correlated with poor prognosis

Qiurong Ye , Huiling Chen, Zonghua Wen, Wenwen Guo, Yongta Huang, Xianglan Mo

Department of Pathology, People's Hospital of Guangxi Zhuang Autonomous Region, Nanning, Guangxi, China

Correspondence to
Dr Xianglan Mo, Department of Pathology, People's Hospital of Guangxi Zhuang Autonomous Region, Nanning, Guangxi, China; moxianglan@21cn.com

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ABSTRACT

Aims The aim of this study is to investigate the expression profiles of cell cycle related proteins in nasal extranodal NK/T cell lymphoma, nasal type (ENKTCL).

Methods The expression profiles of cell cycle related proteins were assessed with a cell cycle antibody array and validated by immunohistochemistry. Correlations between the expression levels of proteins and clinical outcomes of patients with nasal ENKTCL were evaluated.

Results The expression of full length ataxia telangiectasia mutated (ATM) in nasal ENKTCL significantly decreased compared with that in nasal benign lymphoid proliferative disease (NBLPD), but the expression levels of p-ATM, CHK2 and RAD51 significantly increased in nasal ENKTCL compared with that in NBLPD. Kaplan-Meier analysis showed that the expression levels of p-ATM and CHK2 in nasal ENKTCL were inversely related to overall survival (p=0.011 and p=0.025, respectively).

Conclusion Abnormalities in the ATM pathway may play a crucial role in the oncogenesis and chemoradiotherapy resistance of nasal ENKTCL.

INTRODUCTION

Nasal extranodal NK/T cell lymphoma, nasal type (ENKTCL) is a high-grade malignancy and is prevalent in Asia and South and Central America. Nasal ENKTCL accounts for over 70% of ENKTCL. The morbidity of ENKTCL in Guangxi is approximately 49.5% of all mature T and NK cell lymphomas.[1] Most patients with nasal ENKTCL are male and have aggressive clinical course and poor prognosis. Radiochemotherapy is a common treatment for nasal ENKTCL, but it often results in severe adverse side effects and poor outcomes. Therefore, investigating the molecular oncogenesis of nasal ENKTCL is necessary, and a novel and promising therapeutic approach must be developed.

Cell cycle dysregulation is a hallmark of cancer. Cell cycle includes an interphase (M phase) and division periods. The M phase is subdivided into a pre-DNA synthesis phase (G1), DNA synthesis phase (S) and pro-DNA synthesis phase (G2). Cell cycle progression is controlled by a series of cyclin-dependent kinases (CDKs), cyclins, and CDK inhibitors. The three main cell cycle checkpoints are the G1/S, G2/M and metaphase checkpoints. Cell cycle stagnation and cell proliferation are inhibited when cell cycle checkpoints are activated. Our previous studies indicated that the prognosis of ENKTCL in Guangxi is worse than in other regions.[2–4] Morphological observation shows that the mitosis of ENKTCL is frequent,[2] and the suppression of Skp2 expression, which is one of the cell cycle control genes, can suppress ENKTCL cell proliferation and results in cell cycle arrest in the G1 phase.[5] Thus, an abnormality in cell cycle control may play a key role in the development of ENKTCLs. In the present study, we used a cell cycle antibody array to screen differential expression profiles of cell cycle related proteins between nasal ENKTCL and nasal benign lymphoid proliferative disease (NBLPD) samples and verified the expression of these proteins in 49 nasal ENKTCL and 30 NBLPD tissues through immunohistochemistry.

METHODS

Screening the differential expression profiles of cell cycle related proteins in nasal ENKTCL and NBLPD samples with an antibody array

A cell cycle antibody array (Full Moon BioSystems, USA) was used in comparing the expression profiles of cell cycle related proteins in nasal ENKTCL (one type of ENKTCL, nasal type) samples with those in NBLPD samples according to the manufacturer's instruction. The samples were obtained from Kangcheng Biotechnology Company. The array consisted of 60 antibodies against cell cycle markers, namely, 14.3.3 Pan, APC11, APC2, ataxia telangiectasia mutated (ATM), c-Abl, CDC14A phosphatase, CDC6, CDC25C, CDC34, CDC37, CDC47, Cdh1, Cdk1/p34cdc2, Cdk2, Cdk3, Cdk4, Cdk5, Cdk7, Cdk8, Chk1, cullin-1, cullin-2, cullin-3, cyclin B1, cyclin C, cyclin D1, cyclin D2, cyclin E, cyclin E2, E2F-1, E2F-2, E2F-3, glycogen synthase kinase 3b, Ki-67, mitochondria, NuMA, p130, p130cas, p14ARF, p15INK4b, p16INK4a, p18INK4c, p19ARF, p19Skp1, p21WAF1, p27Kip1, p35nck5a, p53, p57Kip2, p73, p73a, p73a/b, proliferating cell nuclear antigen (PCNA), RAD 51, retinoblastoma, retinoblastoma (Phospho-specific Ser608), regulator of cullins (ROC), topo II β, tubulin-a and tubulin-b.

Validation of the expression profiles of cell cycle proteins in nasal ENKTCL and NBLPD samples by immunohistochemistry

A total of 49 patients with nasal ENKTCL and 30 patients with NBLPD were enrolled in this study. All the patients were initially diagnosed with nasal ENKTCL or NBLPD without any other diseases, and all the pathological specimens were reviewed

and reclassified by three experienced pathologists according to the WHO criteria[6] for pathological diagnosis.

The follow-up period for the 41 patients varied from 1 month to 71 months, with a median of 17 months. Twenty-six patients (63%) died during follow-up. Overall survival (OS) was measured from date of diagnosis to date of death or last follow-up visit.

The expression of full length ATM (fATM) (Abcam), its phosphorylated protein p-ATM (Abcam), and CHK2 (Abcam) and RAD51 (Abcam) proteins were detected using EnVision (Dako) according to the manufacturer's instructions. Antigen retrieval was performed using 0.01 M EDTA buffer at pH 9.0 and heating in an autoclave for 5 min. An immunohistochemistry technology was used in staining anti-fATM (1:80), p-ATM (1:200), CHK2 (1:100) and RAD51(1:400) antibodies with an antibody diluent (Dako). Formalin-fixed samples from tonsils were used as positive controls for fATM, p-ATM, CHK2 and RAD51. The primary antibody was substituted with the antibody diluent, which served as the negative control for antibodies.

Tissue sections were evaluated by light microscopy for the determination of fATM, p-ATM, CHK2 and RAD51 positivity. Positive cells were counted in each tissue section. A cut-off point of 10% nuclear staining and/or cytoplasm staining of tumour cells was selected for statistical analysis. Cases with more than 10% of fATM-positive, p-ATM-positive, CHK2-positive and RAD51-positive tumour cells were scored as positive, whereas those with less than 10% of fATM-positive, p-ATM-positive, p-CHK2-positive and RAD51-positive tumour cells were scored as negative. Positive cases were grouped as follows: patients with negative expression or with positive cells with no more than 50% of fATM, p-ATM, CHK2 and RAD51 were considered low expression, whereas those with more than 50% fATM-positive, p-ATM-positive, p-CHK2-positive and RAD51-positive cells were considered high expression.

Statistical analysis

$\chi 2$ and Fisher's exact tests were used for comparing the differences between clinical features and expression of fATM, p-ATM, pCHK2 and RAD51. The correlations between the expression levels of fATM, p-ATM, CHK2 and RAD51 were analysed through Spearman's method. The Kaplan-Meier method was used in the estimation of OS and generation of survival curves. Prognostic parameters were identified by a forward stepwise Cox regression analysis. A value of $p<0.05$ was considered statistically significant.

RESULTS

Cell cycle antibody array screening

Cell cycle antibody array screening showed the differential expression of cell cycle related proteins between samples of NBLPD and nasal ENKTCL were fATM, RAD51, cyclin B and CHK1. The expression levels of fATM and RAD51 in nasal ENKTCL samples significantly differed from those in the NBLPD samples.

Correlation of clinical features and fATM, p-ATM, CHK2 and RAD51 protein expression

Protein expression levels of fATM, p-ATM, CHK2 and RAD51 were validated by immunohistochemistry on the basis of the antibody array results. The expression levels of fATM, p-ATM (figure 1A), CHK2 (figure 1B) and RAD51 in nasal ENKTCL tissues were high in 10 (20.4%), 23 (47%), 24 (49%) and 32 cases (65%), respectively. The expression levels of fATM in the NBLPD tissues were high in 30 cases (100%), but none of the NBLPD samples had high p-ATM, CHK2 and RAD51

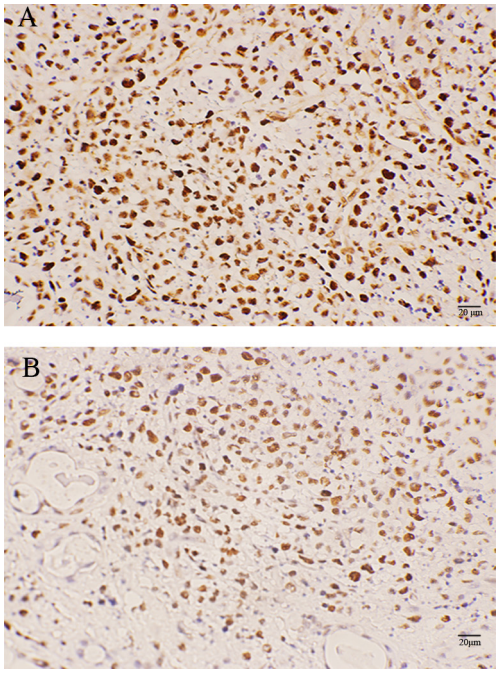

Figure 1 The ATM pathway relative protein expression in nasal extranodal NK/T cell lymphoma, nasal type (ENKTCL). (A) Expression of p-ATM in nasal ENKTCL. Tumour cells were positive for p-ATM. (B) Expression of CHK2 in nasal ENKTCL. Tumour cells were positive for CHK2. Magnification × 400, scale bar is 20 µm.

expression levels. Compared with NBLPD, the expression levels of fATM in nasal ENKTCL significantly decreased ($p=0.000$). Spearman correlation analysis showed a significant negative correlation between the expression levels of fATM and CHK2 in nasal ENKTCL ($r=-0.303$, $p=0.045$) and a positive correlation between the expression levels of p-ATM and CHK2 ($r=0.727$, $p=0.000$). However, a relationship of RAD51 expression with fATM, p-ATM or CHK2 expression ($p>0.05$) wasn't observed in the nasal ENKTCL samples. We also analysed the relation between the expression level of fATM or RAD51 with clinical features of patients with nasal ENKTCL. The expression level of fATM wasn't correlated with sex, clinical stage, B symptoms, lactic dehydrogenase (LDH) level, lymph node involvement or international prognostic index (IPI) group ($p>0.05$), similar to RAD51 except IPI group ($p=0.017$).

Patient survival based on fATM, p-ATM, p-CHK2 and RAD51 expression

The expression levels of p-ATM and CHK2 in nasal ENKTCL were inversely related to survival according to Kaplan-Meier analysis ($p=0.011$, $p=0.025$, respectively). The high-level expression of p-ATM and CHK2 in patients with nasal ENKTCL led to shorter survival as compared with that of patients with low-level expression proteins. The median survival times of patients with high p-ATM and CHK2 expression levels were 6 months (95% CI 3.075 to 8.925) and 7 months (95% CI 4.732 to 9.268), respectively, whereas those of patients with low p-ATM and CHK2 expression levels were both 21 months (95% CI 6.740 to 35.260, 95% CI 10.7 to 31.20, respectively; figure 2A,B). Multivariate analysis showed independent prognostic factors among the included variables, but neither p-ATM nor CHK2 was an independent prognostic factor for nasal ENKTCL.

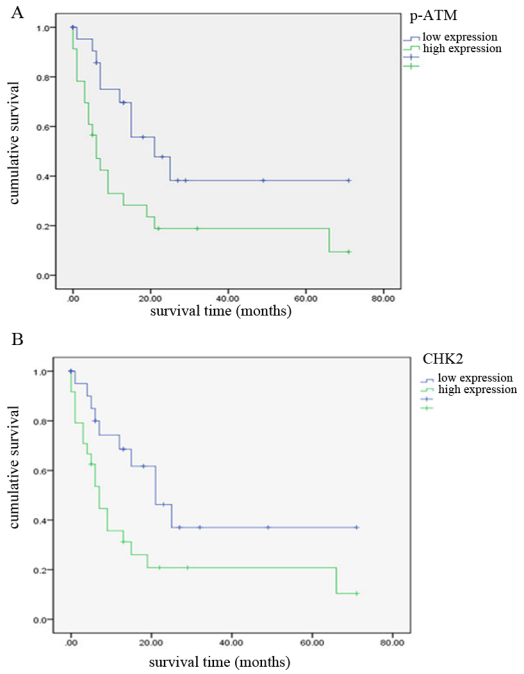

Figure 2 Kaplan-Meier survival analysis in nasal extranodal NK/T cell lymphoma (ENKTCL). (A) Cumulative survival based on expression of p-ATM for 49 patients with nasal ENKTCL. Kaplan-Meier survival analysis showing a significant negative correlation between p-ATM expression and survival in 23 patients who demonstrated high expression compared with 26 patients who demonstrated low expression (p=0.011, Mantel-Cox log rank). (B) Cumulative survival based on expression of CHK2 for 49 patients with nasal ENKTCL. Kaplan-Meier survival analysis showing a significant negative correlation between CHK2 expression and survival in 24 patients who demonstrated high expression compared with 25 patients who demonstrated low expression (p=0.025, Mantel-Cox log rank).

DISCUSSION

Cell cycle antibody array screening showed significant difference in fATM and RAD51 expression between the NBLPD and nasal ENKTCL samples. Immunohistochemistry was used in determining the levels of fATM and RAD51 in 49 nasal ENKTCL and 30 NBLPD tissues. The results revealed that the expression levels of fATM in nasal ENKTCL significantly decreased relative to those in NBLPD, whereas the expression levels of RAD51 significantly increased. The abnormal expression of p27 and Skp2 in the nasal ENKTCL samples was not detected during cell cycle antibody chip screening partially because the protein concentrations of p27 and Skp2 were extremely low.

The ATM signalling pathway plays a key role in DNA damage response and cell cycle checkpoint activation. The ATM gene is located on 11q22-23 and encodes a 350 kDa protein with 3056 amino acids. The ATM protein is a member of the phosphatidylinositol 3- kinase-related kinase family and a serine/threonine kinase. ATM is activated by double-stranded DNA breaks, and the activation procedure involves autophosphorylation and the dissociation of ATM dimers into monomers. The latter process drives Chk2 and p53 phosphorylation and mediates the effects of ATM on DNA repair, cell-cycle arrest and other physiological processes.[7 8] ATM pathway abnormality is associated with a series of human diseases. The low-level expression of the ATM protein is closely related to invasion and worse prognosis in breast cancer.[9 10] A mouse model of pancreatic ductal adenocarcinoma with ATM conditional deletion demonstrates proliferative precursor lesions and fibrotic reaction. In ATM-targeted mice, the transforming growth factor beta(TGFβ)-superfamily signal transduction is changed, epithelial-to-mesenchymal transition is strengthened and survival is shortened. Human pancreatic ductal adenocarcinoma with low ATM expression has poor prognosis.[11] In the current study, we observed the low expression of the fATM protein in nasal ENKTCL that was similar to other human solid malignancies. We also observed that high p-ATM protein expression level is correlated with poor prognosis in nasal ENKTCL. Our results indicate that the ATM pathway participates in the development and progression of nasal ENKTCL.

Chk2, a crucial gene of the ATM signal, is located on 22q12.1 with 22 exons. The CHK2 protein encoded by the Chk2 gene is a serine threonine kinase with 543 amino acid residues and three independent domains. Substrates phosphorylated by CHK2 include proteins associated with DNA repair, cell cycle control, p53 signalling and apoptosis. CHK2 directly participates in early repair of DNA strand breaks by phosphorylating the proteins BRCA1 and BRCA2. CHK2 also participates in resection and repair of base pairs. When a DNA strand breaks, CHK2 arrests the cell cycle at G1/S and G2/M phase until the lesions undergo repair. Gene mutation or abnormal Chk2 expression is linked to a range of human cancers. Havranek and his colleagues[12] found that germline Chk2 mutations affecting protein coding sequences lead to a moderately increased risk of lymphoma and is linked to undesirable outcomes. The expression of CHK2 and p-CHK2 in papillary thyroid cancer tissues is significantly increased compared with that in cancer-adjacent tissues. The overexpression of p-CHK2 in primary tumour tissues is related to tumour progression.[13] An early study showed that the mutation of the Chk2 gene is rarely involved in the development of selected lymphomas.[14] Our present investigation suggests that the expression of CHK2 is upregulated in nasal ENKTCL and associated with unfavourable prognosis. The results imply that changes in the ATM/CHK2 signal pathway contribute to the oncogenesis of nasal ENKTCL.

The Rad51 gene, a key gene in the ATM pathway, is located on 15q15.1 with 13 exons. This gene encodes a protein with multiple functions and crucial roles in DNA repair, homologous recombination repair and gene stability maintenance. Additionally, the protein inhibits innate immune response.[15] Alterations in Rad51 are related to a number of human cancers. In the peripheral blood mononuclear cells of adult T cell lymphoma (ATLL), the expression of RAD51 is significantly increased compared with the expression of asymptomatic human T-cell leukemia virus 1 (HTLV-1) carriers. The expression level of RAD51 is positively correlated with viral load.[16] The RAD51 may be a key factor of HTLV-1's oncogenesis of ATLL. The expression of RAD51 in oesophageal and colorectal adenocarcinomas is positively correlated with lymph node metastasis and poor prognosis.[17 18] Cervical cancers with high-level RAD51 expression have poor prognosis,[19] but some studies showed that cancers with low-level RAD51 expression also have poor prognosis. The expression levels of RAD51, ATM and BRCA1 mRNA in breast cancers are lower than those in benign breast diseases. Spearman's correlation analysis showed a positive correlation between the expression levels of ATM, BRCA1 and RAD51. Low-level ATM expression in breast cancer is significantly correlated with clinical stage, vessel invasion, perineural invasion, malignant features and estrogen receptor (ER) status. Low RAD51 expression is correlated with lymph node metastasis, histopathological

grade and progesterone receptor (PR) status.[20] RAD51 has a low expression in non-small cell lung cancer and is correlated with decrease in survival.[21] Our study shows that the expression of RAD51 protein is significantly increased in nasal ENKTCL compared with that in NBLPD, but we failed to detect a correlation between RAD51 expression and prognosis in nasal ENKTCL. Large-sample studies should be conducted to identify the role of RAD51 in predicting the prognosis of patients with nasal ENKTCL.

In addition to promoting the development of human tumour, the ATM signalling pathway is closely related to chemoradiotherapy and immunotherapy resistance in human cancers. The high-level expression of p-ATM, CHK2, p-CHK2 and p-53 in non-small cell lung cancer is related to cisplatin resistance. The downregulation of ATM expression in non-small cell lung cancer can increase the toxicity of cisplatin, enhance apoptosis and decrease the size of transplantation tumours.[22] In pancreatic cancer, the suppression of ATM can improve the efficacy of immune checkpoint blockade.[23] The small-molecule inhibitors of the ATM/CHK2 pathway can significantly increase the sensitivity of chemoradiotherapy in human cancer.[24] RI-1, a small molecule inhibitor of RAD51 protein, can reduce the growth of xenografts of cervical cancer cells (HeLa and SiHa). Cervical cancer cells treated with RI-1 are more susceptible to cisplatin and ionising radiotherapy than those not treated with RI-1.[25] The downregulation of RAD51 expression in multiple myeloma cells can increase the sensitivity of cancer cells to melphalan treatment.[26] Curcumin can lead to DNA damage in sensitive lymphoma cells with RAD51-dependent homologous recombination and induce apoptosis in a caspase 3-dependent manner. Curcumin increases the susceptibility of lymphoma cells to various DNA-damaging drugs.[27]

CONCLUSIONS

The expression profiles of fATM, p-ATM, CHK2 and RAD51 proteins in the nasal ENKTCL samples are different from those in the NBLPD samples. The high expression levels of p-ATM and CHK2 in nasal ENKTCL are correlated with a poor prognosis. A positive correlation was found between p-ATM and CHK2 expression levels in the nasal ENKTCL samples. Although we could not find a significant correlation between fATM and p-ATM expression levels, a trend of a negative association between these proteins was observed. Our results indicate that the abnormal activation of the ATM signalling pathway may play a crucial role in development and drug resistance of nasal ENKTCL. Thus, investigating the role of the ATM pathway in the radiochemotherapy resistance of nasal ENKTCL is necessary, and a novel, promising treatment must be developed.

Take home messages

- Nasal extranodal NK/T cell lymphoma, nasal type (ENKTCL) is a high-grade malignancy and is prevalent in Asia and South and Central America. Nasal ENKTCL accounts for over 70% of ENKTCL.
- The expression of p-ATM, CHK2 and RAD51 was significantly increased in nasal ENKTCL compared with that in nasal benign lymphoid proliferative disease.
- High expression levels of p-ATM and CHK2 in nasal ENKTCL are correlated with a worse prognosis.

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Contributors Conceived and designed the experiments: QY, XM. Performed the experiments: QY, HC, ZW, WG, YH and XM. Analysed the data: QY. XM. Wrote the paper: QY, XM.

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Competing interests None declared.

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ORCID iD

Qiurong Ye http://orcid.org/0000-0001-5829-189X

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