STAT3 And PD-L1 Are Negatively Regulated By ATM And Impact On The Prognosis Of Triple Negative Breast Cancer Patients With Low ATM Expression

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Research Article

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Abstract

Introduction: Triple negative breast cancer (TNBC) is known for its aggressive behaviors and lacking of effective treatment. Programmed cell death ligand-1 (PD-L1) inhibitor has just been approved for using in the management of advanced TNBC. To accurately screen TNBC sensitive to anti-PD-L1 treatment and to explore the feasibility of the ataxia-telangiectasia mutation protein (ATM) inhibitor combined with PD-L1 inhibitor, radiotherapy and chemotherapy, we focus on whether ATM participates in the regulation of PD-L1 and affects the prognosis of patients through c-Src, signal transducer and activator of transcription 1&3 (STAT1 and STAT3).

Materials and methods: We used immunohistochemical staining to explore the relationship of ATM with c-Src, STAT1, STAT3, PD-1 / PD-L1, Tumor-infiltrating lymphocytes (TILs), as well as other clinicopathologic features in 86 pathological stage II TNBCs. Their impact on prognosis was also explored.

Results: We found ATM expression was negatively correlated with STAT1, STAT3, PD-L1, TILs and CD8 cells in TNBC. STAT1 up-regulates the expression of PD-L1. Only in TNBC with ATM low expression, PD-L1 was an independent negative prognostic factor, while STAT3 was an independent prognostic factor for improved prognosis. Again, only in low ATM group, the phosphorylation of tyrosine at position 419 of c-Src (p-c-src Y419) induced overexpression of STAT3.

Conclusion: TNBC with low ATM expression is more likely to benefit from anti-PD-L1 inhibitors. The feasibility of ATM functional inhibitor combined with immune checkpoint blockade therapies in the treatment of TNBC is also worthy of further exploration. Our study suggests that STAT3 has different impacts on tumor progression in different tumors.

Introduction

Triple-negative breast cancer (TNBC) is a type of breast cancer characteristically lacking expression of estrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor receptor 2 (HER2). It known for its clinical aggressive behaviors and there is no effective treatment modality yet^[1]. Chemotherapy has currently been the mainstay of management, but the curative outcome is not satisfactory. The fact that Tumor-infiltrating lymphocytes (TILs) in TNBC is higher than that in other tumor types prompts people to believe TNBC may have higher immunogenicity, and may subject to immunotherapy^[2-4]. Immune checkpoints are accessory molecules that either promote or inhibit T cell activation. Program cell death ligand-1 (PD-L1) is considered to be the ligand of programmed cell death (PD-1), and the collaborative activity of both could limit the killing ability of cytotoxic T lymphocytes to tumors, thus promoting tumor growth^[6, 7]. The inhibition of PD-1/PD-L1 signaling pathway has proven to a potential therapy that significantly improves the survival of patients with metastatic solid cancers^[5]. Antibodies targeting this pathway have shown promising results in many malignancies^[8, 9], but so far with little success in breast cancer^[10]. Detection of PD-L1 expression by immunohistochemistry has been

used in many clinical trials to select breast cancer patients suitable for the immunotherapy. Studies have shown that expression of PD-L1 is more prominent in primary tumor than in metastasis, therefore detection of the gene expression in primary tumor becomes the first choice for TNBC^[11]. Other study reports that atezolizumab, a humanized monoclonal antibody against PD-L1, in combination with Nab-paclitaxel extends progression-free survival in patients with unresectable, locally advanced or metastatic TNBC with expression of PD-L1^[12].

The ataxia-telangiectasia mutation protein (ATM) gene is a cell cycle checkpoint gene located on chromosome 11q22-23 and plays an important role in maintaining gene stability and repair of DNA double-strand breaks. At another hand, ATM also could induce cell cycle arrest and apoptosis^[13] by regulating the phosphorylation of p53. Alterations in the ATM gene are the main cause of the rare autosomal recessive disease, ataxia telangiectasia (AT), which accounts for 1% of the population and is responsible for the high (3-5 times) incidence of breast cancer in AT patients^[14, 15]. When DNA damage occurs, ATM decompositions inactive dimers into active monomers through automatic phosphorylation at ser1981, which in turn activates downstream signaling pathways and enables DNA repair to occur^[16, 17]. The outcome of ATM on tumor progression is bidirectional, and could go on completely opposite directions. For example, the inactivation of ATM could lead to the obstruction of Fas-induced apoptosis pathway, which promotes progression of lymphoma^[18]. However, lack of ATM may also inhibit hypoxia induced angiogenesis of tumor cells, thus reducing the viability of tumor cells^[19]. ATM inhibitors are studied in clinical research attempting for tumor therapy. The most common sample is for tumor cells resistant to radiotherapy and to certain chemotherapeutic drugs (such as platinum), in which the inhibitors can improve tumor sensitivity by inhibiting the repair of DNA damage^[20, 21].

Zhang et al.'s study linked the loss of ATM to overexpression of PD-L1. They found that deletion of ATM in pancreatic cancer could induce c-Src phosphorylation and activate signal transducer and activator of transcription 1 (STAT1) through c-src/TBK/T1IFN pathway, and finally up-regulate the expression of PD-L1 [28]. Their study indicates that DNA damage is associated with the regulation of immune checkpoints, and suggest ATM inhibition in combination with immune checkpoint blocking and radiation could be an efficacious treatment strategy for pancreatic cancer. Furthermore, DNA damage response alterations (including ATM) were associated with a significantly higher measurable response rate to PD-1/PD-L1 blockade in urothelial cancer^[22]. In addition to STAT1, studies have found that blockade of signal transducer and activator of transcription 3 (STAT3) lead to discrete PD-L1 expression^[23], and c-Src also could induce STAT3 activation^[24]. It is unknown whether ATM participates in the regulation of PD-L1 and impact on the prognosis of patients through c-Src, STAT1 and STAT3 in TNBC.

In this study, we employed immunohistochemistry to explore the relationship between ATM and c-Src, STAT1, STAT3, PD-1 / PD-L1, TILs, FOXP3+, CD8+ cells as well as clinicopathologic features, to explore the relationship ATM with PD-L1 and these specific signal molecules of TNBC, to accurately screen TNBC patients who may be beneficial from anti-PD-L1 therapy, and to evaluate the feasibility of combining ATM inhibitor with PD-L1 inhibitor, radiotherapy or chemotherapy of TNBC patients.

Materials And Methods

Ethics statement

All human breast tissues were collected with written consent from patients prior to participation in the study. The protocols for collection and analysis of the samples were approved by the Institutional Review Board of Tianjin Medical University Cancer Institute and Hospital, in accordance with the current revision of the Helsinki Declaration.

Human breast cancer specimens

The 86 cases used in this study were all the available pTNM stage $\geq$ TNBC excisional samples from Tianjin Cancer Hospital from August 2014 to December 2016. Cases with equivocal HER2 status (2+) but lack of FISH confirmation, and cases without sufficient primary invasive carcinoma for study were excluded. The patients ranged in age from 29 to 83, with a median age of 53. Among the 86 specimens, there were 77 cases of invasive ductal carcinoma (IDC), 6 cases of IDC with apocrine metaplasia, 1 case of lobular carcinoma, 1 case of apocrine adenocarcinoma, and 1 case of squamous cell carcinoma. None of the patients received neoadjuvant chemotherapy before surgery, but all received adjuvant chemotherapy and radiotherapy after surgery.

Immunohistochemical procedure

Immunohistochemical analysis on formalin fixed tissue sections was performed with an avidin-biotin system using a standard protocol. Briefly, 4 μ m tissue sections on coated slides were heated for antigen retrieval, pretreated with a 3% solution of hydrogen peroxide for 10 minutes, rinsed and incubated with 10% normal goat serum as a blocking agent. The sections were then incubated sequentially with primary monoclonal antibody (Supplementary Materials Table S1), biotinylated secondary antibody and avidin-peroxidase conjugate. All steps were preceded by rinsing of sections with PBS (pH 7.6). The chromogen was DAB. PD-L1 (SP142) IHC was conducted using 4 μ m-thick full sections. IHC staining was performed on a Roche Benchmark XT automatic immunohistochemical staining system with antibody detection using the OptiView DAB IHC Detection Kit (Ventana Medical Systems), according to the manufacturer's manual^[25].

Evaluation of immunohistochemistry

CD8 and Foxp3 were stained on the cell membranes and nuclei of tumor stromal infiltrating lymphocytes respectively. Ten fields were randomly selected under high magnification ($\times 400$). The number of positive cells in each field was calculated, and then the average number of positive cells in the 10 fields was calculated. The median (50) of tumor stroma CD8+ T lymphocyte were used as the critical value to divide the cases into high group and low group. The case was considered FOXP3+ Tregs enrichment when the average number of positive cells ≥ 10.5 (median). Staining of ATM, STAT1, STAT3 and p-c-srcY419 was scored semi-quantitatively as negative and weak (1), intermediate (2) and strong (3) in intensity at nucleus (ATM) or cytoplasm (STAT1, STAT3 and p-c-srcY419). The percentage of stained cells was

recorded, and then an H-score (Staining intensity $\times$ Percentage of positive cells) was calculated^[26]. Cases with H-score $\geq$ median (for ATM, H-score $\geq$ 60; for STAT1, H-score $\geq$ 20; and for p-c-srcY419, H-score $\geq$ 20) were considered high expression for corresponding molecules and $<$ median were considered low expression for corresponding molecules. Since the median of STAT3 H-score was 0, H-score $\geq$ 1% was defined as STAT3 positive. TILs were assessed according to the 2014 International TILs Working Group criteria^[27], dividing into two groups according to the median (10%). Staining of Ki67 was performed according to the methods in our previous publication^[28]. The results of Ki67 were based on identification of nuclear stain and divided into two groups according to the median (60%). PD-1 staining was considered positive in cyto-membrane of TILs^[29]. All the above results were determined independently by two pathologists with more than 5 years of working experience who independently read the films by blind method.

PD-L1 IHC was assessed in all 86 cases by two dedicated study pathologists, who independently read the films by blind method, with training in breast cancer diagnosis following the IMpassion130 trial criteria^[30]. PD-L1 positive cases show PD-L1 expression on stromal tumor-infiltrating immune cells occupying $\geq$ 1% of the tumor area.

Statistics

SPSS 22.0 software was used for statistical analysis. Spearman rank correlation test was used for the association analyses between two skew-distributed markers. Kaplan-Meier method was used for overall survival (OS) and invasive disease free survival (DFS) analyses; Log-rank test was used for survival test. Cox proportional risk model was used for multivariate survival analysis. $P < 0.05$ indicates a statistically significant difference.

Result

ATM was negatively correlated with STAT1, STAT3, PD-L1, TILs and CD8 cells in TNBC.

Using the raw scores derived from analysis of the immunohistochemistry (Fig. 1), our data showed that the expression of ATM was negatively correlated with the expression of STAT1 (rs=-0.227, $P=0.036$), STAT3 (rs=-0.239, $P=0.026$), PD-L1 (rs=-0.213, $P=0.049$), TILs (rs=-0.279, $P=0.009$) and CD8+ cells (rs=-0.239, $P=0.027$), but no correlation was found with PD-1+ cells and the clinicopathological features (Table. 1). The positive or negative of “rs” means positive correlation or negative correlation respectively.

STAT1 but not STAT3 up-regulates expression of PD-L1, TILs and some specific markers in TNBC

Again, using the raw scores of immunohistochemistry in Fig. 1 (Table. 2), the data showed that the expression of STAT1 was positively correlated with the expression of PD-L1 (rs=0.422, $P < 0.001$), TILs (rs=0.312, $P=0.003$), FOXP3+ (rs=0.312, $P=0.004$), CD8+ cells (rs=0.413, $P < 0.001$) and STAT3 (rs=0.457,

$P < 0.001$). However, no correlation between STAT3 expression was found with that of PD-L1 ($r_s = 0.023$, $P = 0.832$), TILs ($r_s = 0.057$, $P = 0.603$), FOXP3+ ($r_s = 0.058$, $P = 0.595$) and CD8+ cells ($r_s = 0.103$, $P = 0.345$). The positive or negative of “rs” means positive correlation or negative correlation respectively.

Age, TILs, Ki67 index, TNM stage, p-c-srcY419, STAT1, STAT3, TILs, PD-1, FOXP3+ and CD8+ cells were included in multivariate analysis for PD-L1 status in TNBC. Univariate analysis showed that high STAT1 {OR (95% CI): 4.798 (1.824–12.616); $P = 0.001$ }, TILs {OR (95% CI): 7.500 (2.313–24.320); $P = 0.001$ } and CD8+ {OR (95% CI): 2.500 (1.024–6.106); $P = 0.044$ } were associated with high PD-L1 level, while high ATM {OR (95% CI): 0.253 (0.101–0.634); $P = 0.003$ } was associated with lower PD-L1 level (Fig. 2). Multivariate analysis showed that high STAT1 {OR (95% CI): 5.895 (1.820–19.091); $P = 0.003$ }, high TILs {OR (95% CI): 11.432 (2.862–45.662); $P = 0.001$ } and higher TNM stage (c VS a+b) {OR (95% CI): 3.073 (1.025–9.216); $P = 0.045$ }, were independent predictors for high PD-L1 in TNBC (Fig. 2).

Only in tumors with ATM low expression that PD-L1 are independent negative and STAT3 are positive prognostic factors, respectively

Sixty-five of the 86 pTNM stage III TNBC cases have complete follow-up information from 12 to 63 months, with a median follow-up time of 44.5 months. There were 28 patients relapsed and 14 patients died (Supplementary Materials Table S2).

STAT3, but not PD-L1, had significant association with DFS ($P = 0.011$ and $P = 0.122$, respectively) and OS ($P = 0.054$ and $P = 0.163$, respectively) in all 65 patients (Supplementary Data Fig. S1). In TNBC with low ATM, increased PD-L1 expression significantly reduced DFS of the patients ($P = 0.026$, Fig. 3a); however this impact of PD-L1 on DFS was not found in TNBC with high ATM expression ($P = 0.525$, Fig. 3b). The increased PD-L1 expression had no significant impact on the OS of patients with either low ($P = 0.761$, Fig. 3c) or high ATM expression ($P = 0.332$, Fig. 3d). In the low ATM TNBC, positive expression of STAT3 was associated with prolonged DFS ($P = 0.005$, Fig. 3e) and OS ($P = 0.021$, Fig. 3g). However these impacts were not found in ATM high group ($P = 0.296$, Fig. 3f for DFS and $P = 0.652$, Fig. 3h for OS).

Univariate analysis showed that STAT3, p-c-srcY419 and PD-L1 were significantly associated with DFS of patients with low expression of ATM. However, in the high expression group, no factors analyzed in the study had significantly impact on prognosis (Table 3). Univariate analysis showed that only STAT3 affected OS in patient with ATM low expression, and again all parameters analyzed did not show significant association with OS in ATM high group. Multivariate analysis proved that expression of PD-L1 was an independent prognostic factor for reduced DFS in patients with TNBCs showing low ATM expression {HR (95% CI): 4.577 (1.250 – 16.764); $P = 0.022$, Table 3} but not in those with high ATM expression. STAT3 expression, but not high p-c-srcY419 expression, was an independent prognostic factor for prolonged DFS in TNBC with low ATM expression {HR (95% CI): 0.205 (0.067–0.624); $P = 0.005$, Table 3}, but not in the high ATM expression group. STAT3 positive expression was the only

independent prognostic factor for prolonged OS in patient with low ATM expression {HR (95% CI): 0.186 (0.037– 0.925); P = 0.040, Table 4}, but not in the high ATM expression group.

Table 1

Correlation between ATM and STAT1, STAT3, PDL-1, tumor infiltrating lymphocytes (TILs) and their some specific markers, as well as other pathological features in all 86 cases of pTNM stage II TNBC.

Indicators	ATM		
	M(Q _R) ^f	<i>r_s</i>	<i>P</i>
Age	53(14)	0.113	0.301
Ki67 index (%)	60(45)	-0.009	0.936
pTNM(IIc VS IIa+IIb)	-	-0.103	0.344
STAT1 ^a	20(100)	-0.227	0.036
STAT3 ^a	0(50)	-0.239	0.026
p-c-srcY419 (H-score) ^a	20(40)	0.006	0.956
PD-1 ^b	-	-0.182	0.093
PDL-1 ^c	0(1)	-0.213	0.049
TILs ^d	10(25)	-0.279	0.009
FOXP3+ ^e	10.5(15)	-0.076	0.487
CD8+ ^e	50(80)	-0.239	0.027
a: Calculated by H-score (Staining intensity×Percentage of positive cells)			
b: PD-1 staining was considered positive in cytomembrane of TILs, while the other cases of our study were considered negative.			
c: Calculated by IC score: the PD-L1 expression on stromal tumor-infiltrating immune cells occupying the proportion (%) of the tumor area.			
d: Scored using the 2014 international TILs working group standard			
e: Average number of positive cells in 10 fields at high magnification(×400)			
f: Expressed by median (interquartile range)			

Table 2

the relationship between STAT1 & STAT3 and PDL-1, tumor infiltrating lymphocytes and their some specific markers, as well as other pathological features in all 86 cases of pTNM stage I TNBC.

Indicators	STAT1			STAT3	
	M(QR) ^f	r _s	P	r _s	P
Age	53(14)	-0.093	0.393	-0.039	0.721
Ki67 index (%)	60(45)	0.125	0.252	-0.019	0.865
pTNM(Ic VS Ia+Ib)	-	-0.077	0.481	-0.185	0.088
STAT1 ^a	20(100)	-	-	0.457	<0.001
STAT3 ^a	0(50)	0.457	<0.001	-	-
p-c-srcY419 (H-score) ^a	20(40)	0.239	0.027	0.234	0.030
PD-1 ^b	-	0.189	0.081	0.121	0.268
PDL-1 ^c	0(1)	0.422	<0.001	0.023	0.832
TILs ^d	10(25)	0.312	0.003	0.057	0.603
FOXP3+ ^e	10.5(15)	0.312	0.004	0.058	0.595
CD8+ ^e	50(80)	0.413	<0.001	0.103	0.345
a: Caculated by H-score (Staining intensity×Percentage of positive cells)					
b: PD-1 staining was considered positive in cytomembrane of TILs, while the other cases of our study were considered negative.					
c: Caculated by IC score: the PD-L1 expression on stromal tumor-infiltrating immune cells occupying the proportion (%) of the tumor area.					
d: Scored using the 2014 international TILs working group standard					
e: Average number of positive cells in 10 fields at high magnification(×400)					
f: Expressed by median (interquartile range)					

Table 3

The effect of STAT1, STAT3, PDL-1, TILs and their some specific markers as well as other clinicopathological features, on the invasive disease-free survival (iDFS) of ATM-low TNBC patients analyzed by univariate and multivariate analysis

Indicators	Univariate analysis			Multivariate Analysis		
	HR	95%CI	<i>P</i>	HR	95%CI	<i>P</i>
Age {≥53(median) VS <53}	0.923	0.334-2.550	0.877			
p-c-srcY419 (H score ≥20 VS <20)	0.307	0.097-0.974	0.045			
STAT1 (H score ≥20 VS <20)	0.739	0.268-2.041	0.560			
STAT3 (Positive VS Negative)	0.244	0.082-0.722	0.011	0.205	0.067-0.624	0.005
Ki67 index (≥60% VS <60%)	1.476	0.524-4.156	0.461			
pTNM(c VS a+b)	1.442	0.521-3.988	0.481			
TILs (≥10% VS <10%)	2.071	0.466-9.199	0.339			
PD-1(Positive VS Negative)	1.007	0.343-2.953	0.990			
PDL-1(Positive VS Negative)	3.769	1.057-13.441	0.041	4.577	1.250-16.764	0.022
FOXP3+ ^a (≥10.5 VS <10.5)	1.227	0.344-3.388	0.693			
CD8+ ^a (≥50 VS <50)	0.864	0.307-2.429	0.781			
a: Average number of positive cells in 10 fields at high magnification(×400)						

Table 4

The effect of STAT1, STAT3, PDL-1, TILs and their some specific markers as well as other clinicopathological features, on the overall survival (OS) of ATM-low TNBC patients analyzed by univariate and multivariate analysis

Indicators	Univariate analysis			Multivariate Analysis		
	HR	95%CI	P	HR	95%CI	P
Age { ≥ 53 (median) VS < 53 }	0.729	0.182-2.916	0.655			
p-c-srcY419 (H score ≥ 20 VS < 20)	0.542	0.129-2.272	0.402			
STAT1 (H score ≥ 20 VS < 20)	0.350	0.083-1.468	0.151			
STAT3 (Positive VS Negative)	0.186	0.037-0.925	0.040	0.186	0.037-0.925	0.040
Ki67 index ($\geq 60\%$ VS $< 60\%$)	3.949	0.791-19.726	0.094			
pTNM(c VS $a+b$)	1.334	0.332-5.354	0.685			
TILs ($\geq 10\%$ VS $< 10\%$)	2.376	0.292-19.343	0.419			
PD-1(Positive VS Negative)	0.891	0.212-3.739	0.874			
PDL-1(Positive VS Negative)	1.248	0.298-5.229	0.762			
FOXP3+ ^a (≥ 10.5 VS < 10.5)	1.813	0.432-7.608	0.416			
CD8+ ^a (≥ 50 VS < 50)	0.433	0.108-1.735	0.237			
a: Average number of positive cells in 10 fields at high magnification($\times 400$)						

Low expression of ATM and phosphorylation of tyrosine at position 419 of c-Src induces overexpression of STAT3, but not STAT1, and improves PFS of patients. To explore the regulatory mechanism of STAT3 and PD-L1, and to analyze why STAT3 and PD-L1 only impact on prognosis in TNBC with low expression of ATM, we analyzed the relationship between p-c-src Y419 and STAT1, STAT3, PD-L1, TILs and some specific tumor immune cell markers at different ATM expression levels. We found in TNBC with low expression of ATM, p-c-src Y419 was only positively associated with STAT3 ($rs=0.356$, $P=0.022$, Table 5), but not significantly with other factors including STAT1 ($rs=0.064$, $P=0.693$, Table 5). However in tumors with high expression of ATM, p-c-src Y419 was positive correlated with STAT1 ($rs=0.426$, $P=0.004$, Table 5) and TILs ($rs=0.335$, $P=0.025$, Table 5), but not significantly with other factors including STAT3 ($rs=0.141$, $P=0.357$, Table 5)

Table 5

The relationship between p-c-src Y419 and STAT1, STAT3, PDL-1, TILs and some specific tumor immune cell markers at different ATM expression levels of TNM stage II TNBC

p-c-srcY419^a						
	ATM low (N=41)			ATM high (N=45)		
Indicators	M(Q _R) ^f	r _s	P	M(Q _R) ^f	r _s	P
Ki67 index (%)	60(32.5)	0.012	0.941	60(50)	-0.133	0.383
STAT1 ^a	30(110)	0.064	0.693	10(67.5)	0.426	0.004
STAT3 ^a	5(70)	0.356	0.022	0(30)	0.141	0.357
PDL-1 ^c	1(1)	-0.261	0.099	0(5)	0.056	0.716
TILs ^d	20(20)	0.145	0.365	10(15)	0.335	0.025
FOXP3+ ^e	11(13.5)	0.232	0.145	10(15)	0.087	0.568
CD8+ ^e	64(119.5)	0.206	0.196	40(50.5)	0.266	0.077
a: Calculated by H-score (Staining intensity×Percentage of positive cells)						
b: PD-1 staining was considered positive in cytomembrane of TILs, while the other cases of our study were considered negative.						
c: Calculated by IC score: the PD-L1 expression on stromal tumor-infiltrating immune cells occupying the proportion (%) of the tumor area.						
d: Scored using the 2014 international TILs working group standard						
e: Average number of positive cells in 10 fields at high magnification(×400)						
f: Expressed by median (interquartile range)						

In TNBC with low ATM expression, high expression of p-c-srcY419 was significantly associated with a better DFS than that of low expression (P=0.032, Fig. 4a), although it is no proven to be independent at multivariate analysis (Table 3) and did not show value in the impact on OS (P=0.395, Fig. 4c). In ATM high expression group, p-c-srcY419 expression was not associated with improved DFS or OS at univariate (Fig. 4b & 4d) and multivariate analysis (Table 3 & 4).

Discussion

In this study, we found that ATM can negatively regulate the expression of PD-L1 and STAT3 in TNBC. Zhang et al. found that inhibition of ATM enhances the activity of c-SRC/TBK1 pathway and thus increase the transcription of tumoral Type I interferon (T1IFN), which activate STAT1, and thus increasing the expression of PD-L1^[31]. Our results are consistent with the findings of Zhang et al. ^[28] and Ramana

CV et al.^[32], in which STAT1 up-regulates the levels of PD-L1 and induces certain inflammatory cell infiltrates. However, STAT3 does not have similar functions in TNBC. At another hand, this study did not establish the correlation between tyrosine phosphorylation at position 419 of c-Src and ATM expression level in TNBC. This suggests that ATM down-regulation may negatively regulate cGAS / STING / TBK signal pathway^[33] and activate IFNs, so as to activate STAT1 and STAT3 at the same time, and then up-regulate the levels of TILs, CD8 + T cells and PDL1. However, negative ATM regulation of STAT1 and STAT3 through other signal pathways cannot be excluded.

Low expression of ATM seems to sensitize the impact of STAT3 on improving prognosis of TNBC patients. In other studies it has been shown that STAT3 predicts adverse prognosis of a variety of tumors. The proposed mechanisms include promoting growth and proliferation^[34], reducing sensitivity of tumor cells to radiotherapy^[35], inhibiting autophagy^[36], promoting tumor angiogenesis^[37], promoting tumor invasion and metastasis^[38] and enhancing tumor immune suppression^[39]. However, some recent studies have reported the inhibitory effect of STAT3 on tumor progression. Ferguson's study suggests that STAT3 controls NF- κ B-induced IL-8 expression by sequestering NF- κ B within the cytoplasm, thereby inhibiting IL-8-mediated myeloid tumor infiltration and tumor vascularization and enhance tumor progression^[40]. The study results of Pencik et al also suggest the loss of STAT3 signaling disrupts the ARF–Mdm2–p53 tumor suppressor axis bypassing senescence and occurs with high frequency in PCa metastases^[41]. Our results showed that STAT3 expression is significantly associated with improved PFS and OS, and is proven to be an independent prognostic factor only for TNBC with low ATM expression. Since both NF κ B pathway^[42] and p53 pathway^[43] are regulated by ATM, the antitumor effect of STAT3 may be related to the low expression of ATM. Our results inferred that the regulatory role of ATM on p53 may be bidirectional. At one hand, the low expression of ATM reduces phosphorylation of p53, therefore some tumor cells with unrepaired mutations may avoid apoptosis and survive. In another hand, low expression of ATM up-regulates the expression of STAT3, which can induce apoptosis of tumor cells through ARF – MDM2 – p53 tumor suppressor axis. In tumors expressing low ATM, the role of ATM in promoting apoptosis by phosphorylating p53 is lost. At this time, STAT3 is particularly important for the removal of mutant tumor cells. This explains the sensitization of ATM down-regulation to the antitumor effect of STAT3. In TNBC with low expression of ATM, the role of p-c-srcY419 is similar to that STAT3. Its overexpression prolongs PFS, but not proven to be an independent factor affecting yet. c-Src kinase is one of the kinases involving in the activation of STAT3^[44], indicating that the antitumor effect of p-c-srcY419 is likely to couple with STAT3 in TNBC. In the cases with low ATM expression, p-c-srcY419 selectively up regulates STAT3, rather than STAT1, which obviously will further strengthen the effect of low ATM expression on the antitumor effect of STAT3. In addition, our results show that in cases with high expression of ATM, the effect of p-c-src Y419 is opposite and does not associated with improved prognosis. STAT3 does not up regulate the expression of PD-L1 inducing antitumor immunosuppression, which will obviously further strengthen the antitumor effect of STAT3. Low expression of ATM enhances the impact of PD-L1 on poor prognosis. ATM is the main switch molecule that directly senses DNA double strand break damage and initiates multiple DNA damage signal response pathways^[45–46]. It plays an

extremely important role in DNA homologous recombination repair and induces apoptosis of cells containing irreparable mutations. When ATM expression is low or with functional loss, tumor cells may enter the cell proliferation cycle without correct repair due to DNA double strand breaks caused by radiotherapy and some chemotherapeutic drugs (such as platinum), and the cumulative mutations of tumor will continue to increase. At this stage, the tumor is more sensitive to anti-tumor immunity, and become more sensitive to the effect of immune checkpoints (such as PD-1 / PD-L1). Up to now, it is only our hypothesis based on the existing data, and confirmation by rigorous signal pathway research is demanded. In addition, our results also suggest that ATM with low expression or loss of function may be more sensitive to immune checkpoint blockade therapies, such as PD-L1 inhibitors.

In summary, our study indicated that STAT3 positively and PD-L1 negatively impact on the prognosis of TNBC patients with low ATM expression. TNBC with low expression of ATM is more likely to benefit from anti-PD-L1 treatment. The feasibility of ATM functional inhibitor combined with immune checkpoint blockade therapies in the treatment of TNBC is also worthy of further exploration. Our study also suggests that STAT3 have different impact on tumor progression in different tumors. The use of STAT3 inhibitors needs to consider the molecular signal transduction in different tumors. This study once again shows the complexity and compensation mechanism of ATM functions, and also suggests that the combined use of ATM inhibitors and other antitumor drugs may benefit more tumor patients.

Declarations

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Compliance with Ethical Standards

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Conflict of interest

The authors have no relevant financial or non-financial interests to disclose.

Author Contributions

All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by Yuan-Ming Song, Xiao-Long Qian, Guang-Shen Gao and Xiao-Jing Guo. The first draft of the manuscript was written by Xiao-Jing Guo and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

Data Availability

The datasets generated during and/or analysed during the current study are available in the Supplementary Information (SI) Table S2.

Ethics approval

This study was performed in line with the principles of the Declaration of Helsinki. Approval was granted by the Institutional Review Board of Tianjin Medical University Cancer Institute and Hospital (Date: March 27, 2020/No:Ek2020146).

This article does not contain any studies with animals performed by any of the authors.

Consent to participate

Informed consent was obtained from all individual participants included in the study.

Consent to publish

The authors affirm that human research participants provided informed consent for publication of the images in Fig. 1a, 1b, 1c, 1d, 1e, 1f, 1g, 1h, 1i, 1j, 1k, 1l, 1m, 1n, 1o, 1p.

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Figures

Figure 1

Examples of immunohistochemical staining of all molecules expressed in TNBC tissues in this study (200×).

a. ATM low (H-score: 0); b. ATM high (H-score: 150); c. p-c-srcY419 low (H-score: 0); d. p-c-srcY419 high (H-score: 210); e. STAT1 low (H-score: 0); c. STAT1 high (H-score: 240); g. STAT3 negative; h. STAT3 positive (H-score: 80); i. PD-1 negative; j. PD-1 positive; k. PDL-1 negative; l. PDL-1 positive (IC score: 20%); m. FOXP3+ low (3/HP); n. FOXP3+ high (60/HP); o. CD8+ low (5/HP); p. CD8+ high (180/HP).

Figure 2

Univariate and multivariate analyses of multi-factors on PDL-1 status of all 86 pathological TNM stage III TNBC cases

Figure 3

The effects of PDL-1 and STAT3 on the prognosis of pathological stage III TNBC patients analyzed by Kaplan-Meier survival curve under different ATM status.

a. The effect of PDL-1 on DFS in ATM low patients; b. The effect of PDL-1 on DFS in ATM high patients; c. The effect of PDL-1 on OS in ATM low patients; d. The effect of PDL-1 on OS in ATM high patients; e. The effect of STAT3 on DFS in ATM low patients; f. The effect of STAT3 on DFS in ATM high patients; g. The effect of STAT3 on OS in ATM low patients; h. The effect of STAT3 on OS in ATM high patients. (DFS: invasive disease free survival; OS: overall survival)

Figure 4

The effect of p-c-srcY419 on the prognosis of pathological stage III TNBC patients analyzed by Kaplan-Meier survival curve under different ATM status.

a. The effect of p-c-srcY419 on DFS in ATM low patients; b. The effect of p-c-srcY419 on DFS in ATM high patients; c. The effect of p-c-srcY419 on OS in ATM low patients; d. The effect of p-c-srcY419 on OS in ATM high patients.

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