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Review Article

TOX gene: a novel target for human cancer gene therapy

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Abstract: Thymocyte selection-associated high mobility group box factor (TOX) is a member of an evolutionarily conserved DNA-binding protein family and is expressed in several immune-relevant cell subsets. TOX encodes a nuclear protein of the high-mobility group box superfamily. It contains a DNA-binding domain, which allows it to regulate transcription by modifying local chromatin structure and modulating the formation of multi-protein complexes. Previous studies have shown that TOX play important roles in immune system. More recently, several studies have described TOX expression is frequently upregulated in diverse types of human tumors and the overregulation often associates with tumor progression. Moreover, TOXis involved in the control of cell apoptosis, growth, metastasis, DNA repair and so on. In this review, we provide an overview of current knowledge concerning the role of TOX in tumor development and progression biology function. To our knowledge, this is the first review about the role of thisnew oncogene in tumor development and progression.

Keywords: TOX, oncogene, cancer, proliferation, apoptosis

Introduction

TOX (Thymocyte selection-associated HMG bOX) genes represent a novel gene family and encode a novel nuclear DNA binding protein belonging to a large superfamily of HMG (high mobility group)-box family. TOX proteins consist a small subfamily of proteins, including TOX1, TOX2, TOX3, and TOX4. Although they shared similar structures, different member of TOX family plays different biological roles. TOX1 was originally identified by microarray as a thymic transcript that was highly upregulated in $CD4^+CD8^+$ double positive (DP) thymocytes by Wilkinson B et al. TOX1 is proved to be a crucial regulator in immune system differentiation. However, the biological roles of the other members of TOX family remain largely unspecified. TOX3 is predominantly expressed in brain neurons and breast while TOX4 is recently proved to be a DNA interacting protein with unknown biological role.

Recently, emerging evidence has shown that TOX genes are aberrantly expressed or mutated in various diseases, especially in several different kinds of malignancies, such as lung cancer, breast cancer, gastric cancer, lymphomas and leukemia. In addition, TOX family members were also involved in non-tumor diseases, such as pulmonary tuberculosis and HIV infection. In some cases, TOX acted as an oncogene, controlling cancer cells physiology via promoting oncogenic signaling. Among them, expressions and roles of TOX1 and TOX3 in malignancies were largely studied while studies on TOX2 and TOX4 are relatively limited. TOX members were also proved to be potential diagnostic or prognostic markers in some malignancies, such as breast cancer and cutaneous lymphomas. However, the roles and molecular mechanisms of TOX in malignancies remain unspecified.

In this review, we summarize the current information on TOX, focusing on their biological roles and their involvement in human malignancies.

The structure of TOX genes and proteins

TOX gene family members are located on four different human chromosomes. TOX1, originally identified as KIAA0808, is located at chromosome 8q12.1 in human and on chromosome 4 A1 in mice. TOX2 is located on 20q13.12 with 12 exons [1, 2]. TOX3, also known as TNRC9 (trinucleotide-repeat-containing 9), is located

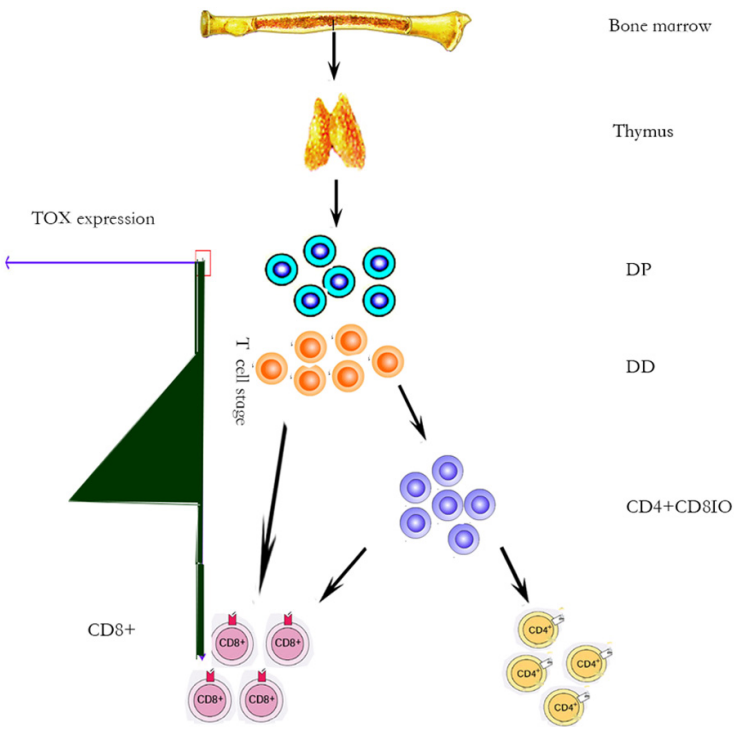

Figure 1. TOX expression in T cell development in the thymus during positive selection. Downregulation of CD4 and CD8 receptors in DP cells yield a double dull (DD) phenotype. Re-expression of CD4 receptor in DD results in $CD4^+CD8^{lo}$ cells. $CD4^+CD8^{lo}$ are precursors of both $CD4^+CD8^-$ (CD4SP) and $CD4^-CD8^+$ (CD8SP) thymocytes. CD8SP can derive either from $CD4^+CD8^{lo}$ or directly from DD cells. TOX is induced to unregulated when DP cells were selected to DD cells. TOX were significantly up-regulated at the $CD4^+CD8^{lo}$ stage. After thymocytes differentiated into mature SP cells, TOX returned to baseline levels.

on 16q12 and consists of seven exons. TOX3 was first identified in brain in a screen for transcripts containing trinucleotide (CAG) repeat expansions in 1997 [3]. TOX3 protects neurons from death by inducing Ca^{2+}-dependent transcription from different cytoprotective promoters in neurons [4, 5]. TOX3 is also a co-factor of CREB and CBP. TOX4 is located on 14q11.2 with 9 exons. TOX4, also known as migration-inducing protein 7 or epidermal Langerhans cell protein LCP1, interact with a phosphatase complex involved in cell cycle progression from mitosis into interphase [6]. In addition, similar to other HMG box proteins, TOX4 protein is proved to be involved in the process of DNA repair damaged by platinum anticancer drugs [6].

TOX proteins belong to a large superfamily of HMG (high mobility group)-box family and contain a DNA-binding domain (the HMG-box) [7]. TOX proteins include a small subfamily of proteins, which include TOX1, TOX2, TOX3, and TOX4 [8]. The TOX subfamily members are highly conserved among vertebrates [2]. Similar to other HMG-box proteins, the sequences of the TOX family members contain an HMG-box DNA binding domain, which comprises three helices folding into an L-shaped structure. The DNA binding domains between different TOX family members are nearly identical. In addition, the N-terminal domain is fairly conserved while the C-terminal domain is family-member specific. Similar to other HMG proteins, TOX seem to operate by bending DNA, thereby altering chromatin structure and modifying the accessibility of transcription factors to DNA [9]. However, the genomic binding sites of TOX remain unspecified. Further studies are needed to understand how this DNA binding factor is targeted to specific regions of DNA.

Expression patterns and biological roles of TOX

TOX1 is proved to be a crucial regulator in immune system differentiation while the biological roles of the other members of TOX family remain largely unspecified. In this section, we will discuss the expression patterns and biological roles of TOX family, focusing on the expressions and roles of TOX1 in immune system differentiation and maturation.

TOX1 gene is most abundantly expressed in the thymus. It is also highly expressed in the liver and brain. However, TOX1 is poorly expressed or even absent in other tissues, including heart, kidney, lung, muscle, skin, intestine, spleen stomach and testis [1]. TOX1 is expressed in many subsets of immune cells, suggesting its significant roles in immune system, including development of CD4 T cells and NK cells, as well as lymph node organogenesis [10-12].

TOX1 is transiently upregulated in thymus during β-selection and positive selection of devel-

Table 1. Clinical relevances of TOX

Cancer	Expression or gene abnormality	Function in the clinic	References
Breast cancer	Downregulated and hypermethylated	TOX hypermethylation improves diagnosis; TOX3 and LOC643714 predict adverse outcome.	[21, 33, 35]
Lung cancer	Downregulated and hypermethylated	Not mentioned	[21]
Cutaneous lymphoma	Upregulated	Molecular marker for diagnosis of mycosis fungoides; High TOX mRNA levels correlated with increased risks of disease progression and disease-specific mortality.	[36-41]
Gastric cancer	Not investigated	TOX3 associated with favorable prognosis	[42]
Leukemia	Downregulated and deleted	Not mentioned	[43, 44]
Central neural lymphoma	Downregulated and deleted	Not mentioned	[47]

oping thymocytes [7]. Accumulating evidence has proved that TOX1 plays a critical role in the generation of all T lineage subsets, including CD4 and CD8 thymocytes. In the absence of TOX1 (TKO), the development of CD4 T cells was blocked, suggesting its important role in CD4 T cell lineage development [10]. TOX-/- (TKO) mice blocked all CD4 T lineage cells, including not only production of conventional CD4 thymocytes, but also development of NKT and FOXP3+ regulatory T cells as well. However, CD8SP T cells developed in TKO mice. These results emphasize that the $CD4^+$ SP transitional stage of development is not necessary for all CD8 T cell development [10, 13]. In addition, transgenic mice that express TOX1 (TOX-transgenic [Tg]) in the majority of thymocytes showed an increased CD8 SP thymocytes and decreased CD4 SP thymocytes [10]. There might exist another pathway for CD8 but not CD4 T cell development (**Figure 1**).

TOX1 was also highly expressed during in vitro NK differentiation and down-regulation of TOX1 led to a decreased population of natural killer (NK) cells, suggesting that TOX1 plays a critical role in human NK cell development [14]. Furthermore, Tox-/- hematopoietic stem cells can differentiate into NK cell precursors, but the NK cell precursors do not differentiate further. Thus TOX1 is considered to play a critical role in immature NK cells [15]. In addition, TOX1 was proved to play key roles in differentiation of cultured $CD34^+$ human cord blood precursor cells into NK cells [14].

In addition, TKO mice lacked lymph nodes and had a significant decrease in the frequency and size of Peyer's patches, suggesting its role in lymph node organogenesis. LTi cells were proved to be the central hematopoietic system-derived orchestrators of lymphoid tissue organogenesis [16]. TOX1 is broadly induced and reciprocally controls classical NK and lymphoid tissue-inducer (LTi) cells [17]. TOX1-/- mice also lack identified fetal and adult LTi cells, explaining the cause of the absence of Peyer's patches and lymph node [15].

While TOX1 is proved to be a crucial regulator in immune system differentiation, little is known about the expressions and roles of other TOX family members. The role of TOX2 in humans remains unclear. The rat ortholog of TOX2 with 100% HMG-box domain homology (GCX-1) is primarily expressed and functions in the hypothalamic-pituitary-gonadal axis of reproduction [1]. A recent study has shown that TOX2 is highly expressed in mature NK cells and is upregulated during the differentiation of NK cells from human umbilical cord blood (UCB)-derived $CD34^+$ cells [2]. Furthermore, silence of TOX2 expression inhibited the early differentiation of NK cells and overexpression of TOX2 promoted UCB $CD34^+$ cells to differentiate into mature NK cells [2]. TOX3 is predominantly expressed in brain neurons. It is also highly expressed in breast. In addition, breast cancer expresses higher levels of TOX3 than the normal breast tissue [4, 18]. The biological role of TOX4 remains to be elucidated. Its N-terminus domain contains a strong transcription activator and it was recently proved to be a platinated-DNA interacting protein [6, 19].

TOX and cancer

Increasing evidence has demonstrated that TOX gene family members are aberrantly expressed or mutated in various human diseases, especially in many kinds of malignancies. In this section, we will discuss the current findings of TOX in various human diseases, focusing on their roles in malignancies (**Table 1**).

TOX and breast cancer

It is now well known that aberrant methylation of hundreds of genes is prevalent during cancer development [20]. Gene silencing through aberrant promoter CpG island hypermethylation is the most frequent epigenetic abnormality observed in various malignancies. In a genome-wide comparison of DNA methylation, the promoter CpG islands of TOX1 are hypermethylated in 43% of breast tumors while it was unmethylated and in the distant normal breast tissue. In addition, in vitro studies proved that TOX1 was completely or partially methylated in three out of four breast cancer cell lines. The expression of TOX1 in the methylated cell lines is downregulated compared to the unmethylated cell line, indicating that TOX1 is epigenetically silenced by promoter hypermethylation [21]. In conclusion, TOX1 was hypermethylated in breast cancers but not in the adjacent normal tissue, suggesting it might be a potential novel tumor biomarker. Another study proved that TOX2 was unmethylated in normal cells but it was methylated in 23% breast (n=80) tumors. Furthermore, expression of two novel TOX2 transcripts was silenced in methylated breast cancer cells [21].

Recent genome-wide association studies (GWAS) have identified several single-nucleotide polymorphisms (SNPs) related with breast cancer risk [22]. TOX3 gene is located on 16q12, a region commonly lost in breast cancers and recently proved as the risk of breast cancer [23]. Several polymorphisms existed in the TOX3 gene, including the SNP rs3803662, rs12443621, and rs805154. Among them, SNP rs3803662 was proved to have a strong association of breast cancer [24-29]. No association was found between the rs12443621 or rs3803662 alleles and breast cancer risk [24, 25, 28-31]. However, the role of the germline polymorphisms in the TOX3 remains unspecified [29]. Although it was previously reported in one study that TOX3 expression was not associated with prognosis of breast cancer patients [32]. Another study showed that the expression levels of TOX3 and/or LOC643714 associated with the progression of breast cancer, depending on the subtype and developmental stage of the tumor [33]. TOX3 might act as a tumor suppressor gene since the risk allele rs3803662 is significantly associated with lower expression of TOX3 in breast cancer [34]. In addition, knockdown of TOX3 expression increased cellular proliferation in breast cancer [35].

TOX and lung cancer

In a genome-wide comparison of DNA methylation between normal and tumor cells, the promoter CpG islands of TOX1 were also methylated in 20% lung cancers cell lines, whereas the distant normal lung tissue from lung cancer patients were unmethylated. In addition, in methylated lung cancer, the TOX1 transcripts expression was silenced [21]. It was considered that TOX1 was silenced through CpG hypermethylation in lung cancer, which provide a possible mechanism for the development lung cancer. TOX2 were methylated in 28% lung (n=190) tumors while it was unmethylated in normal cells. Expression of TOX2 transcripts was significantly decreased in methylated lung cancer cells [21].

TOX1 and cutaneous lymphoma

Primary cutaneous lymphomas (PCLs) are a heterogeneous group of neoplasias that are characterized clonal proliferations of neoplastic T or B lymphocyte in the skin with no evidence of extracutaneous disease at the time of diagnosis [36, 37]. PCLs can be classified into to cutaneous T-cell lymphomas (CTCL) and cutaneous B-cell lymphomas (CBCL). In CTCL, there are indolent subtypes such as mycosis fungoides (MF) and lymphomatoid papulosis, whereas other CTCL subtypes have a more pejorative prognosis such as Sézary syndrome (SS) and CD30-lymphomas. Recently, Youwen Zhou et al. proved that TOX1 was highly expressed in early MF skin biopsies in immunohistochemistry and immunofluorescence [38]. The authors further found that thicker MF lesions such as plaques and tumors expressed higher TOX1 levels than thinner patches, suggesting its association with MF progression. High TOX1 mRNA levels correlated with increased risks of disease prognosis [39]. In addition, SohshiMorimura et al. demonstrated higher levels of TOX1 mRNA in SS using immunohistochemistry [40]. Furthermore, TOX1 is a direct target of microRNA-223, which could reduce cell growth and clonogenic potential of MF [41]. In conclusion, TOX1 was highly expressed in some CTCLs, such as MF and SS, suggesting its role in the pathogenesis of CTCL.

TOX3 and gastric cancer

In a recent study, the effect of TOX3 rs3803662 on survival of gastric cancer patients was investigated. TOX3 rs3803662 was proved to be associated with a significantly favorable effect among diffuse-type gastric cancer patients. Thus it was concluded that TOX3 rs3803662 might play an important role in the prognostic outcome and treatment of gastric cancer [42].

TOX1 and leukemia

Most childhood acute lymphoblastic leukemia (ALL) can be cured, but the prognosis is dismal in patients who relapse after treatment. Genome-wide DNA copy number analyses on 61 pediatric patients with relapsed ALL identified copy number alterations of TOX1 (8q12.1) at relapse [43]. In contrast to acute lymphoblastic leukemia in children, adult cases are associated with a very poor prognosis. Recurrent deletions of TOX1 were also seen in relapse samples of adult ALL, suggested that TOX might be associated with relapsing ALL [44].

TOX1 and primary central nervous system lymphomas

Primary central nervous system lymphoma (PCNSL) is a very aggressive rare brain tumor characterized by accumulation of malignant cells. The prognosis of PCNSL is typically worse without treatment and the incidence of PCNSL is increasing [45, 46]. In patients with newly diagnosed PCNSL, the molecular characterization of TOX1 showed biallelic deletions in copy number abnormalities (CNA) [47].

TOX as prognostic markers

Given that deregulated expressions of TOX were identified many cancers, it provides an attractive approach for cancer management. First of all, TOX can be used as biomarkers for cancer diagnosis and prognosis. By monitoring TOX status in an individual tumor, the risk of cancer development and progression can be predicted, as well as the prognosis of the cancer. For example, early MF diagnosis is a major challenge in the clinical practice. TOX1 was highly expressed in early MF skin biopsies and TOX1 mRNA levels had good discriminatory power for MF, demonstrated by an area under the curve (AUC) value of 0.87. For MFdiagnosis, TOX1 mRNA levels showed sensitivity of 90.3% 75.0% specificity of 75.0%, when cutoff was set at 2.99. In addition, high TOX1 mRNA levels correlated with increased risks of disease progression and disease-specific mortality [39]. In diagnosing breast cancer, the combination of EGFR5 or TOX1 hypermethylation showed a sensitivity of 92% and specificity of 92% and accuracy of 93%. The combination of DPYS or TOX1 hypermethylation showed a sensitivity of 88%, specificity of 96% and accuracy of 91% [48]. Expression levels of TOX3 and/or LOC643714 were proved to affect the prognosis of breast cancer. Tumors with the risk allele had shorter overall survival (OS) and high TOX3 and/or LOC643714 correlated with positive lymph nodes in breast cancer [33]. The TOX3 rs3803662 CT/TT genotype associated with better survival among diffuse-type gastric cancer patients, serving as an independent prognostic marker [42].

Mechanisms of TOX deregulation in cancers

As mentioned above, TOX was frequently deregulated in a variety of human malignances. Deregulation of gene expression can be caused by two mechanisms: one is genetic alteration, namely gene mutation or loss of heterozygosity (LOH), and the other is epigenetic event, such as CpG island promoter hypermethylation. In this section, we will discuss the upstream regulations of deregulation TOX gene expression in diseases.

One possible mechanism is that TOX1 itself is mutated and subsequently resulted in its aberrant expression. Evidence to support this notion is that recurrent deletions of TOX1 was seen in relapse samples of adult ALL and childhood ALL [43, 44]. In addition, in patients with newly diagnosed PCNSL, TOX1 showed biallelic deletions in copy number abnormalities (CNA) [47]. In a mutation screen study for TOX3, four mutations were identified (three missense, one in-frame deletion of 30 base pairs) in six primary tumors out of 133 breast tumors [49].

The other upstream regulation might due to the epigenetic change in TOX genes. Epigenetic change refers to a stable change in gene expression that can be inherited through subsequent cell divisions, without a change in DNA

sequence. Epigenetic regulation plays an important role during carcinogenesis and tumor development. Epigenetic changes include DNA methylation and histone modifications [50, 51]. DNA methylation results in transcriptional repression, which occurs mainly in CpG islands of the promoter region [52, 53]. The promoter CpG islands of TOX1 were hypermethylated in both lung cancers and breast cancer, which silenced TOX1 transcripts expression [21]. Thus TOX1 was supposed to be silenced through CpG hypermethylation in cancers [54-56]. However, the methylation status in other cancers remains unknown. Further studies are needed to elucidate the possible relationship between the expression of TOX1 and promoter hypermethylation in other malignancies. MicroRNAs (miRs) are endogenous 18-25 nucleotide non-coding RNAs that target specific mRNA for translational repression or degradation [57, 58]. MiRs can regulate various cellular functions, including cell survival, proliferation, migration, invasion and metastasis and expression levels of miRs is often deregulated in cancer. TOX1 is a direct target of microRNA-223, which could reduce cell growth and clonoge nic potential of MF [41]. The study provides a novel sight involving a miR-TOX1 axis to understand the mechanism of TOX deregulation.

Conclusions and future perspectives

TOX was initially identified through its association with T lymphocyte differentiation. TOX proteins contain a small subfamily of proteins, including TOX1, TOX2, TOX3, and TOX4. Different member of TOX family plays different biological and pathological roles. It is known that TOX is a DNA binding protein; however the genomic binding site of TOX is unknown. Future investigations are needed to identify genomic binding sites of TOX, to understand how it is targeted to specific regions of DNA. In addition, although emerging evidence has shown that TOX was deregulated in many tumors, the functional roles of TOX in tumors remain unspecified, which also requires future investigations.

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Disclosure of conflict of interest

None.

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