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Review

Immunotherapy in Acute Leukemias: Implications and Perspectives Using Wt1 Antigen

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Abstract. *The WT1 gene encodes a transcription factor involved in regulation of many cellular processes, including proliferation, differentiation, mRNA processing and apoptosis, besides acting as a transcription repressor of growth factors and their receptors' genes. This gene is expressed at high levels in several types of cancers, including acute leukemias. In this regard, many studies have identified WT1 protein as a tumor antigen, considered a target molecule for clinical application in human acute leukemias. Immunotherapy using WT1 antigen has been effective in stimulating immune responses against leukemic cells. Regarding adoptive immunotherapy, the use of dendritic cells (DCs) for the WT1-specific cytotoxic T cells generation proved to be efficient in the development and maintenance of immunologic cells. Therefore, these therapeutic methods, that provided enthusiasm for moving ahead, highlight several opportunities and challenges to be used in clinical practice for managing acute leukemias.*

Wilms' tumor is derived from pluripotent embryonic renal precursors (1) and is histologically composed by stromal, epithelial cells and undifferentiated mesenchymal cells (2). It may arise as a result of the inactivation of both alleles of the *wilms tumor 1* (*WT1*) gene, located on chromosome 11p13 (3), being considered a tumor suppressor gene. Interestingly, in breast cancer (BC), soft-tissue sarcoma, high tumor-stage in testicular germ-cell tumors, head and neck squamous cell carcinoma (HNSCC) and leukemias, *WT1* mRNA transcription levels represent an important factor correlated with poor prognosis (4, 5).

In fact, it is known that the *WT1* gene is expressed at high levels in several solid tumor types, including brain (astrocytes), HNSCC, thyroid, esophagus, lung, breast, pancreas (ductal cancer), colon cancers, as well as in bone sarcomas and soft tissues (6).

Leukemias are a heterogeneous group of diseases with clonal disorders of hematopoietic cells, which differ in etiology, clinical presentation, course and response to therapy (7). Casually, they are classified in lymphoid or myeloid, according to cell lineage commitment, and in acute or chronic, regarding cellular differentiation and course of the disease.

In acute leukemias, the morphological examination of blood and bone marrow (BM) frequently reveal accumulation of immature neoplastic cells or blasts, which can infiltrate organs, such as liver, spleen, lymph nodes, meninges, brain and skin. In BM, they proliferate and produce symptoms associated with bone marrow failure like anemia, neutropenia and thrombocytopenia (8).

Immunotherapy has been considered a promising method in the treatment for cancer, especially leukemia. It aims to induce or amplify the antitumor immunity (9). In oncology, this modality of therapy has been well-established; however, continuing advances in genetic engineering concerning antibodies and T cells should strengthen its clinical impact in

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the upcoming years (10). Considering that *WT1* acts as an oncogene in some malignant neoplasms, including leukemias, this article focuses on reviewing the immunotherapeutic strategies of this transcription factor on acute leukemias.

WT1 Transcription Factor

The *WT1* gene encodes a 55 kDa transcription factor (11), which has a carboxyl-terminal region containing four zinc-finger domains that mediate DNA binding. The amino terminal end, which is rich in prolines and glutamines, contains transcriptional activation domains, similar to other transcription factors (1).

This transcription factor is involved in the regulation of many cellular processes, including proliferation, differentiation, mRNA processing and apoptosis (12, 13), and may act as a repressor of growth factor genes' transcription, such as platelet-derived growth factor (PDGF), colony-stimulating factor 1 (CSF-1), insulin-like growth factor 2 (IGF-2) and transforming growth factor beta (TGF-β), as well as receptor genes for growth factors, such as IGF-1 receptor (IGF-1R), epidermal growth factor receptor (EGFR) and vitamin D receptor (14).

Furthermore, it may also act by mediating expression on other genes, such as retinoic acid receptor alpha (*RAR-α*), v-myb avian myeloblastosis viral oncogene homolog (*c-myb*), v-myc avian myelocytomatosis viral oncogene homolog (*c-myc*), ornithine decarboxylase (*ODC*) and v-myc avian myelocytomatosis viral oncogene neuroblastoma derived homolog (*N-myc*) (15).

The *WT1* gene includes ten exons (12) and can encode more than 36 different proteins depending on RNA edition (16). Several transcriptional changes can occur; however, two alternative splicings are prevalent, by means of which the four major isoforms are encoded (5). Two of these isoforms differ from each other due to the presence or absence of 17 amino acids (17 AA (+) and 17 AA (–)) encoded by exon 5, exclusively in mammals. This set of amino acids is added in the middle of the 17 AA isoform and is essential for the WT1 interaction with prostate apoptosis response factor 4 (Par-4), a transcriptional coactivator (12).

The other two isoforms are differentiated due to the inclusion or exclusion of amino acids lysine, threonine and serine (KTS) (KTS (+) and KTS (–)), which are located between the third and fourth zinc-finger binding site (17), encoded by exons 9 and 10, respectively (18). The normal ratio of WT1 isoforms is 2 KTS (+):1KTS (–) and this can be considered stable during fetal development and throughout life (17).

It was shown that, among the four predominant isoforms, the 17 AA (+) KTS (+) and 17 AA (+) KTS (–) were the ones most expressed during primary leukemia and exerted its anti-apoptotic function by stabilizing the potential of mitochondrial membrane in leukemia cells; although, the expression of these isoforms was reduced in solid tumors (5).

In adult humans, *WT1* expression is very low and limited to some tissues, including kidney (podocytes), ovaries (granulosa cells), testis (*Sertoli* cells), spleen, myoepithelial progenitor cells and normal hematopoietic progenitor cells (16, 19). It can be further expressed in the central nervous system and hematopoiesis-related tissues, such as BM and lymph nodes (20).

Indeed, *WT1* is expressed at low levels in normal BM and restricted to $CD34^+$ cell population (20). Notwithstanding, it is suggested that *WT1* contributes to development of blood cells, since its expression occurs in early hematopoietic precursors and decreases after differentiation (21).

WT1 and Acute Leukemia

Even though the *WT1* gene was originally defined as a tumor suppressor gene, it has been proposed that it holds oncogenic properties in leukemogenesis and tumorigenesis (6, 22). This hypothesis can be justified for several reasons: it was observed that expression is up-regulated in acute myeloid leukemia (AML) and in blast crisis of chronic myeloid leukemia (CML) (18); treatment with antisense-*WT1* oligomers (3) and small-hairpin RNA (WT1-shRNA) (5) promoted growth inhibition of leukemic cell and solid tumors expressing WT1 (23); constitutive WT1 expression in myeloid progenitor cells blocked their differentiation; however, in response to granulocyte colony-stimulating factor (G-CSF), it induces proliferation (6). Moreover, inhibition of WT1 expression resulted in suppressed growth of leukemic cells *in vitro* (22). Otherwise, it was observed that enhanced expression of WT1 promoted motility, suppressed apoptosis and induced leukemia in transgenic mice (3).

WT1 overexpression has been described in leukemias, mostly in AML, allowing for monitoring of minimal residual disease (MRD) (24). In addition, in recent years, some results have been reported involving acute lymphoid leukemia (ALL), although they are still controversial. In pediatric ALL, Boublikova *et al.* (11) demonstrated that T-ALL presented increased WT1 expression compared to B-ALL patients. However, these results were not confirmed in children or in adults (16, 25).

Busse *et al.* (16) demonstrated that WT1 expression levels at diagnosis have no prognostic impact on the clinical outcome in adult ALL. Furthermore, a sudden increase in *WT1* expression in remission patients seems to be associated with an increased risk of relapse (26). These findings were also reported in pediatric patients (27). On the other hand, Boublikova *et al.* (11) reported that low *WT1* expression can be associated with an increased risk of relapse.

Iranparast *et al.* (28) showed a strong correlation between WT1 and vascular endothelial growth factor (VEGF)

expressions in leukemia patients, suggesting that WT1 may regulate neoplasia development and angiogenesis in acute blood cancer.

Considering the genetic mutations presented by ALL patients, it was observed that patients with chromosomal translocation t(4;11), harboring MLL-AF4 fusion proteins, had extremely high WT1 levels compared to other chromosomal abnormalities, such as t(12;21) - TEL-AML1 and t(9;22) - BCR-ABL (11). Contrariwise, Hu *et al*. (27) found a positive correlation between WT1 overexpression and *BCR-ABL* fusion gene, in both ALL and AML pediatric patients.

Therefore, *WT1* mRNA could represent a biomarker for leukemic blast cells (6) and may serve as a quantitative marker in acute leukemias (19). Taken together, these findings highlight a promising target to immunotherapy in leukemias.

WT1 and Immunotherapy

Cancer immunotherapy is considered the fourth cancer therapy after the three major ones: surgery, chemotherapy and radiotherapy. For maximal efficacy, immunotherapy should be started as soon as possible after the diagnosis of cancer and continued as long as possible, so that surgery, chemotherapy and radiotherapy can be performed under conditions of enhanced cancer immunity (3).

Several tumoral antigens, recognized by T cells, have already been identified, based on protein expression pattern, and classified in a few groups (29): antigens resulting from mutations in proto-oncogenes and tumor suppressor genes, such as RAS and p53 proteins (30); tumor-specific shared antigens, such as MAGE-1 (31); overexpressed tumor antigens, such as HER-2/Neu (32); and antigens derived from oncogenic viruses, such as E7 oncoprotein of human papilloma virus (HPV) 16 (33).

Research in cancer immunology has advanced significantly after the characterization of these antigens that may represent potential candidates for cancer immunotherapy. In this regard, 75 representative antigens in cancer have been selected and studied according to the following criteria: therapeutic function; immunogenicity; function of oncogenic antigen; specificity; antigen-positive cells and expression levels; expression in stem cells; number of cancer patients who present the antigen; number of antigenic epitopes and cellular localization of expression of antigen. Cheever *et al*. (34) evaluated these antigens and, although none of them presents all ideal antigen characteristics, WT1 was ranked at the top. This result has initiated the development of immunotherapy using WT1 as the target antigen in cancer.

In general, the identification of WT1 as a tumor antigen is most often associated with acute leukemias, since several studies have shown increased expression in leukemic cells compared to normal hematopoietic cells. Indeed, the viability of leukemic cells can be partly maintained by WT1 expression (35).

Among the cancer immunotherapy strategies, vaccines represent a specific active therapy with massive development prospects. The therapy using WT1 as a vaccine aims to increase the number of specific B and T lymphocytes against the antigen present on tumor cells. The administration of a synthetic cytotoxic T lymphocyte (CTL) WT1 epitope, in combination with an adjuvant, was able to induce a WT1-specific CTL response. The adjuvant activates dendritic cells (DCs) in skin and the WT1 peptide fits into the groove of human leukocyte antigen (HLA) class I molecules on the cell surface. Then, this complex moves to lymph nodes, where they activate $CD8^+$ T lymphocytes that recognize the complex (36).

Hence, the development of vaccines targeting WT1 antigen depends on the characterization of peptides associated with HLA molecules on the cell surface. In this context, different epitopes have been identified with this potential (23, 36).

In an experimental model, animals were sensitized with WT1 9-mer peptide, containing binding domains to HLA class I. It was verified that CTLs were able to destroy tumor cells expressing the antigen. In addition, animals immunized with WT1 peptide were immunogenic, responding successfully against WT1-expressing tumor cells (6).

Moreover, vaccinated animals that had been stimulated with WT1-positive tumor cells were significantly longer survivors than the animals that had been vaccinated with control plasmid or saline (35). Furthermore, HLA-A *0201-restricted CTLs, a type of HLA class I frequent in Caucasians, were produced *in vitro* to recognize WT1 9-mer peptide. It was found that isolated cells from CML patients, HLA-A *0201-negative in chronic phase, were not recognized by specific CTLs for this peptide.

Oka *et al*. (37) have also demonstrated that, through immunization of experimental animals, CTLs against the WT1 protein could discriminate differences in WT1 expression levels between abnormally WT1-overexpressing tumor cells and physiologically WT1-expressing normal cells, thus resulting in tumor cell death without damage to normal tissues.

Several studies in clinical trials have evaluated the safe and effective vaccination of various WT1 peptides in AML (38-40). This approach increased the immune system activation and response against tumor cells (22, 41). Sawada *et al*. (42) have used WT1 peptide vaccination in children with various types of malignancies and observed, in some patients, an increase in $CD8^+$ T cells that recognized WT1 antigen and, consequently, an increase of interferon gamma (IFN-γ) in peripheral blood.

Although CTLs are the most effective immune elements against tumor cells, $CD4^+$ T lymphocyte response is extremely important in the generation and maintenance of antitumor cytotoxic responses. In this context, several groups

have investigated epitopes capable of inducing immune responses *via* class II major histocompatibility antigens (MHC) (43, 44).

Guo *et al*. (44) reported the possibility of inducing specific $CD4^+$ T cells for WT1 20-mer peptide, with restricted cytotoxicity to HLA class II. In this regard, the $CD4^+$ T cells, specific for WT1, played an important role against leukemic cells expressing WT1 and induced $CD8^+$ CTL activity.

On the other hand, Brayer *et al*. (43) have used a combined vaccine capable of inducing responses *via* class I and II MHC, checking their safety, tolerability and immunogenicity. In a protocol involving patients with AML and myelodysplastic syndrome (MDS), the vaccine was well-tolerated and several patients showed clinical improvement; however, it was not possible to detect consistent WT1-specific immune responses.

Sera from patients with AML were tested against the WT1 antigen and 3 out of 18 patients had antibodies directed against the N-terminal region of the protein, suggesting that the immune system recognizes the WT1 antigen (45). These results were also observed in patients with ALL, CML and MDS. The authors detected IgM antibodies against WT1 in 54.8% of patients, compared to 16.2% of healthy controls; IgG antibodies were detected in a similar proportion (46).

Although more studies are needed to confirm the role of WT1 expression in clinical practice, Qi *et al*. (47) suggested that WT1 can also be a potential marker for predicting disease-free survival, relapse or relapse-free survival and progression-free survival in patients with solid tumors. In particular, the administration of natural or modified 9-mer peptide was able to induce regression of breast cancer with colon metastasis, as well as maintained free survival or disease recurrence. In addition, patients with lung cancer showed tumor mass reduction after the second dose of WT1 vaccine (48).

In order to improve the specific immune response to WT1 soluble antigen, adoptive immunotherapy has been proposed in acute leukemias.

WT1 in Adoptive Immunotherapy

Adoptive immunotherapy consists of isolation and infusion of cells with antitumor activity in patients. This property is acquired in *ex vivo* cultured cells, promoting expansion and modification, depending on the clinical design (49).

Immune response targeting of adoptively transferred cells is important to ensure effective and therapeutic action. Particularly, it is initially mandatory to search for tumoral or tumor-associated antigens that can be the therapeutic targets. Moreover, it is necessary to evaluate antigen immunogenicity and its physiological involvement, aiming treatment effectiveness and avoiding tissue toxicity. For this reason, the increased expression of WT1 in acute leukemias suggests a potential target for the adoptive therapy design.

It has been described that presentation of WT1 peptides *via* HLA-A*2402 and HLA-A*0201 is able to generate immune responses of CTLs (50, 51). Various WT1 peptides were reported to induce specific CTLs in a murine experimental model (45). Among tested peptides, the most explored is the WT1 9-mer, presented *via* HLA-A*2402, and the p126 (peptide sequence: RMFPNAPYL) WT1, presented *via* HLA-A*0201 (51, 52).

It is known that peptides binding with high affinity to HLA-A exhibit enhanced immunogenicity (53). Thus, modifications on WT1 peptides were proposed to increase its specificity and immunogenicity. Tsuboi *et al*. (54) found that the substitution of an amino acid at position 2 of WT1 9-mer peptide increased the specificity for HLA-A*2402 and, also, increased the efficiency of inducing a CTL response when compared with the unmodified peptide. In addition, amino-acid substitutions at HLA-A*0201-binding anchor positions in native 9-mer WT1 peptide have more efficiently induced WT1-specific HLA-A*0201-restricted CTL than the natural 9-mer WT1 peptides (55). Essentially, CTLs are very important cells in the immune response against tumor cells, with the activation of their effector mechanisms occurring by recognition of tumor antigen peptides presented *via* MHC class I (56).

In a clinical study (57), transplanted patients with relapsed acute leukemias, CML or MDS received 5 infusions of WT1-specific CTLs derived from matched donors. The results suggested absence of significant treatment-related toxicities and that CTLs could be mobilized to bone marrow and have anti-leukemic activity, due to reduction of leukemic blasts.

Another clinical trial (58) tested WT1-specific CTLs derived from matched donors, generated by DCs infected with adenovirus vector, which encodes a truncated WT1 protein by deletion of the N-terminal region (1-147aa), in a transplanted patient with AML. After four infusions, the presence of WT1-specific CTLs was observed and maintained until the last examination over 2 years. Unfortunately, this patient died due to graft-versus-host disease (GVHD).

Ochi *et al*. (59) produced a retroviral vector (WT1-silencer siTCR) with a specific alpha and beta T cell receptor (TCR) chains for WT1 that promoted the silencing of non-specific TCR chains for WT1 with small hairpin RNA (shRNA) that binds to the constant regions of endogenous TCR of CTLs. The modified human CTLs from leukemia patients successfully lysed autologous leukemia cells but not normal hematopoietic progenitor cells. In a mouse xenograft model, adoptively transferred modified CTLs exerted distinct anti-leukemia efficacy and did not inhibit human hematopoiesis, which have cells with low WT1 expression. Likewise, using the same methodology, no renal injury was observed *in vivo* mediated by modified CTLs (60). These findings were important for establishing the immunotherapy using this methodology, because of safety and efficiency.

In addition to these findings, Fujiwara *et al*. (61) have modified $CD4^+$ T cells and CTLs using the same experimental design. They found that modified $CD4^+$ T cells migrated to leukemia sites and, subsequently, mediated an attraction of WT1-specific CTLs *via* chemotaxis, resulting in leukemia suppression. Importantly, the presence of modified $CD4^+$ T cells was correlated with longer survival and enhanced formation of memory T cells by modified $CD8^+$ T cells. In this context, these authors suggested that adoptive immunotherapy, using modified $CD4^+$ T cell and modified CTLs together, would be clinically advantageous for the treatment of leukemia.

Furthermore, Isao *et al*. (62) administered modified lymphocytes with a retroviral vector MS3-WT1-siTCR, which encodes a specific TCR for WT1 235-243 peptide in patients with AML and MDS. The preliminary results showed transient decline of peripheral abnormal cells that were MDS-related. They also observed a decrease of bone marrow blasts and no toxicity induced by therapy.

In addition, DCs have an important role in the tumor environment. DCs are known as the most potent professional antigen presenting cells (APCs), inducing tumor-specific effector T cells, which can reduce the tumor mass specifically, maintaining innate and adoptive immune responses and promoting immunological memory. In this regard, the use of DCs for the WT1-specific CTLs generation has been assured as an efficient methodology in adoptive immunotherapy (63, 64).

In a clinical phase II study, autologous *WT1* mRNA-electroporated-DCs were administered in 10 patients with AML at high risk of full relapse, treated with polychemotherapy, in order to observe its anti-leukemic effect. Some patients reached complete and some partial remission. The activation of both innate and adaptive immune responses was also noted (65). However, the production of such cells is time consuming and strenuous to be carried out on a large scale.

Sundarasetty *et al*. (66) evaluated the effect of smart dendritic cells (SmartDCs, which means "Self differentiated myeloid-derived antigen presenting cells reactive against tumor DCs"), produced using a vector expressing truncated WT1, stimulated with granulocyte-macrophage colony-stimulating factor (GM-CSF) and infused in mice. Noteworthy, the production of SmartDCs is faster and such cells have higher viability, biodistribution and specific immune response than conventional DCs (67, 68). In this experiment, they observed that the WT1-specific SmartDCs are able to attract WT1 specific human CTLs *in vivo*. In another setting, *ex vivo* combination of human CTLs and SmartDCs inhibited tumor growth by activating the specific response of CTLs to WT1, suggesting a potential use of these cells in leukemia adoptive immunotherapies (66).

Another *in vitro* study, using electroporated DCs with *IFN-α* and *WT1* mRNA, successfully demonstrated a stimulation of antigen-specific CTLs and enhancement of natural killer (NK) cells effector functions, thereby activating the innate and adaptive immunity (69).

Thus, for maintenance of the CTLs population, administration of modified DCs that present WT1 epitopes represents a promising and exciting approach in modern anticancer therapy as it can provide a glimpse into the future of acute leukemia treatment involving adoptive immunotherapy.

Conclusion

Immunotherapy targeting WT1 antigen in acute leukemias has demonstrated specificity and effectiveness, since this immunogenic peptide is present in high concentrations in leukemic cells. Considering vaccine treatment with WT1 peptide, the results were promising in animal models and in clinical studies. Immunization of AML patients led to activation of CTLs and $CD4^+$ T cells and, thereby, increased immune response against tumor cells.

In relation to adoptive immunotherapy, the use of DCs for WT1-specific CTLs' generation proved to be efficient in the development and maintenance of immunologic cells. Furthermore, genetic engineering in the production of WT1-responsive cells also showed an encouraging performance, due to control and plasticity of such cells. Thus, to increase therapeutic effectiveness, developing an adoptive immunotherapy directed to WT1 antigen may consider both tools. In addition, it is important to note that activation of both subpopulations of lymphocytes, CTLs and $CD4^+$ T cells, was significantly important to generate a sustained response and immunological memory. The specificity of adoptive immune transferred cells is mandatory for this type of therapeutic management. Therefore, the identification of tumor antigens or tumor associated antigens, which can be potential therapeutic targets, is necessary. Furthermore, analysis of antigen immunogenicity and their physiological presence is important to ensure effectiveness of treatment and avoid toxicity to normal tissues.

Although several studies have revealed promising results, further investigations are needed to establish and improve these strategies, opening up the potential for a new era of management of acute leukemias.

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References

1 Rivera MN and Haber Da: Wilms' tumour: Connecting tumorigenesis and organ development in the kidney. Nat Rev Cancer *5(9)*: 699-712, 2005.

2 Akpa MM, Iglesias D, Chu L, Thiebaut A, Jentoft I, Hammond L, Torban E and Goodyer PR: Wilms tumor suppressor, wt1, cooperates with microrna-26a and microrna-101 to suppress translation of the polycomb protein, ezh2, in mesenchymal stem cells. J Biol Chem *291(8)*: 3785-3795, 2016.

3 Sugiyama H: Wt1 (wilms' tumor gene 1): Biology and cancer immunotherapy. Jap J Clin Oncol *40(5)*: 377-387, 2010.

4 Oji Y, Miyoshi S, Maeda H, Hayashi S, Tamaki H, Nakatsuka SI, Yao M, Takahashi E, Nakano Y, Hirabayashi H, Shintani Y, Oka Y, Tsuboi A, Hosen N, Asada M, Fujioka T, Murakami M, Kanato K, Motomura M, Kim EH, Kawakami M, Ikegame K, Ogawa H, Aozasa K, Kawase I and Sugiyama H: Overexpression of the wilms' tumor gene wt1 in de novo lung cancers. Int J Cancer *100(3)*: 297-303, 2002.

5 Tatsumi N, Oji Y, Tsuji N, Tsuda A, Higashio M, Aoyagi S, Fukuda I, Ito K, Nakamura J, Takashima S, Kitamura Y, Miyai S, Jomgeow T, Li Z, Shirakata T, Nishida S, Tsuboi A, Oka Y and Sugiyama H: Wilms ' tumor gene wt1-shrna as a potent apoptosis-inducing agent for solid tumors. Int J Oncol *32(3)*: 701-711, 2008.

6 Sugiyama H: Cancer immunotherapy targeting wilms' tumor gene wt1 product. Expert Rev Vaccines *4(4)*: 503-512, 2005.

7 Chang H, Salma F, Yi Q-l, Patterson B, Brien B and Minden MD: Prognostic relevance of immunophenotyping in 379 patients with acute myeloid leukemia. Leukemia Res *28(1)*: 43-48, 2004.

8 Ferrara F: Unanswered questions in acute myeloid leukaemia. Lancet Oncol *5(7)*: 443-450, 2004.

9 Mackall CL, Merchant MS and Fry TJ: Immune-based therapies for childhood cancer. Nature *11(12)*: 1-11, 2014.

10 Mellman I, Coukos G and Dranoff G: Cancer immunotherapy comes of age. J Clin Oncol *29(36)*: 4828-4836, 2011.

11 Boublikova L, Kalinova M, Ryan J, Quinn F, O'Marcaigh A, Smith O, Browne P, Stary J, McCann SR, Trka J and Lawler M: Wilms' tumor gene 1 (wt1) expression in childhood acute lymphoblastic leukemia: A wide range of wt1 expression levels, its impact on prognosis and minimal residual disease monitoring. Leukemia *20(2)*: 254-263, 2006.

12 Morrison AA, Viney RL and Ladomery MR: The post-transcriptional roles of wt1, a multifunctional zinc-finger protein. Biochim Biophys Acta *1785(1)*: 55-62, 2008.

13 Wagner N, Wagner KD, Hammes A, Kirschner KM, Vidal VP, Schedl A and Scholz H: A splice variant of the wilms' tumour suppressor wt1 is required for normal development of the olfactory system. Development *132(6)*: 1327-1336, 2005.

14 Menssen H, Siehl J and Thiel E: Wilms tumor gene (wt1) expression as a panleukemic marker. Int J Hematol *76(2)*: 103-109., 2002.

15 Sugiyama H: Wilms' tumor gene wt1: Its oncogenic function and clinical application. Int J Hematol *73(2)*: 177-187, 2001.

16 Busse A, Gökbuget N, Siehl JM, Hoelzer D, Schwartz S, Rietz A, Thiel E and Keilholz U: Wilms' tumor gene 1 (wt1) expression in subtypes of acute lymphoblastic leukemia (all) of adults and impact on clinical outcome. Ann Hematol *88(12)*: 1199-1205, 2009.

17 Guaragna MS, Cristina A, Britto GD, Bittencourt B, Santos C, Piveta C, Soardi FC, Claudio L, Castro G, Maria V and Belangero S: Frasier syndrome: Four new cases with unusual presentations. Arq Bras Endocrinol Metabol *56(8)*: 525-532, 2012.

18 Huff V: Wilms' tumours: About tumour suppressor genes, an oncogene and a chameleon gene. Nat Rev Cancer *11(2)*: 111-121, 2015.

19 Van Driessche A, Gao L, Stauss HJ, Ponsaerts P, Van Bockstaele DR, Berneman ZN and Van Tendeloo VFI: Antigen-specific cellular immunotherapy of leukemia. Leukemia *19(11)*: 1863-1871, 2005.

20 Yang L, Han Y, Saiz SS and Minden MD: A tumor suppressor and oncogene : The wt1 story. Leukemia *21(5)*: 868-876, 2007.

21 Ellisen LW, Carlesso N, Cheng T, Scadden DT and Haber DA: The wilms tumor suppressor wt1 directs stage-specific quiescence and differentiation of human hematopoietic progenitor cells. EMBO J *20(8)*: 1897-1909, 2001.

22 Di Stasi A, Jimenez AM, Minagawa K, Al-Obaidi M and Rezvani K: Review of the results of wt1 peptide vaccination strategies for myelodysplastic syndromes and acute myeloid leukemia from nine different studies. Front Immunol *6(36)*: 1-6, 2015.

23 Fujiki F, Oka Y, Tsuboi A, Kawakami M, Kawakatsu M, Nakajima H, Elisseeva OA, Harada Y, Ito K, Li Z, Tatsumi N, Sakaguchi N, Fujioka T, Masuda T, Yasukawa M, Udaka K, Kawase I, Oji Y and Sugiyama H: Identification and characterization of a wt1 (wilms tumor gene) protein-derived hla-drb1*0405-restricted 16-mer helper peptide that promotes the induction and activation of wt1-specific cytotoxic t lymphocytes. J Immunother *30(3)*: 282-293, 2007.

24 Inoue K, Sugiyama H, Ogawa H, Nakagawa M, Yamagami T, Miwa H, Kita K, Hiraoka A, Masaoka T and Nasu K: Wt1 as a new prognostic factor and a new marker for the detection of minimal residual disease in acute leukemia. Blood *84(9)*: 3071-3079, 1994.

25 Ibrahim A, Badrawy H and Sayed H: Prognostic implications of expression of the wilms tumor 1 (wt1) gene in acute leukemia (experience from south egypt). Brit J Med Med Res *7(1)*: 61-71, 2015.

26 Ujj Z, Buglyó G, Udvardy M, Beyer D, Vargha G, Biró S and Rejtő L: Wt1 overexpression affecting clinical outcome in non-hodgkin lymphomas and adult acute lymphoblastic leukemia. Pathol Oncol Res *20(3)*: 565-570, 2014.

27 Hu S-Y, Gu W-Y, Chen Z-X, Wang X-L, Cen J-N, He H-L, Chai Y-H and Chen C-S: The significance of detecting wt1 expression in childhood acute leukemias. Ped Hematol Oncol *27(8)*: 581-591, 2010.

28 Iranparast S, Assarehzadegan M-a, Heike Y, Hossienzadeh M and Khodadadi A: Wilms' tumor gene (wt1) expression correlates with vascular epithelial growth factor (vegf) in newly acute leukemia patients undergoing chemotherapy. Asian Pac J Cancer P *15(21)*: 9217-9223, 2014.

29 Van den Eynde BJ and van der Bruggen P: T cell defined tumor antigens. Curr Opin Immunol *9(5)*: 684-693, 1997.

30 Gjertsen MK, Bjørheim J, Saeterdal I, Myklebust J and Gaudernack G: Cytotoxic cd4+ and cd8+ t lymphocytes, generated by mutant p21-ras (12val) peptide vaccination of a patient, recognize 12val-dependent nested epitopes present within the vaccine peptide and kill autologous tumour cells carrying this mutation. Int J Cancer *72(5)*: 784-790, 1997.

31 Traversari C, van der Bruggen P, Luescher IF, Lurquin C, Chomez P, Van Pel A, De Plaen E, Amar-Costesec a and Boon T: A nonapeptide encoded by human gene mage-1 is recognized on hla-a1 by cytolytic t lymphocytes directed against tumor antigen mz2-e. J Exp Med *176(5)*: 1453-1457, 1992.

32 Fisk BB, Blevins TL, Wharton JT and Ioannides CG: Identification of an immunodominant peptide of her-2/neu protooncogene recognized by ovarian tumor-specific cytotoxic t lymphocyte lines. J Exp Med 181(6), 1995.

33 Ressing ME, Sette A, Brandt RM, Ruppert J, Wentworth Pa, Hartman M, Oseroff C, Grey HM, Melief CJ and Kast WM: Human ctl epitopes encoded by human papillomavirus type 16 e6 and e7 identified through *in vivo* and *in vitro* immunogenicity studies of hla-a*0201-binding peptides. J Immunol *154(11)*: 5934-5943, 1994.

34 Cheever MA, Allison JP, Ferris AS, Finn OJ, Hastings BM, Hecht TT, Mellman I, Prindiville SA, Viner JL, Weiner LM and Matrisian LM: The prioritization of cancer antigens: A national cancer institute pilot project for the acceleration of translational research. Clin Cancer Res *15(17)*: 5323-5337, 2009.

35 Tsuboi A, Oka Y, Ogawa H, Elisseeva OA, Li H, Kawasaki K, Aozasa K, Kishimoto T, Udaka K and Sugiyama H: Cytotoxic t-lymphocyte responses elicited to wilms' tumor gene wt1 product by DNA vaccination. J Clin Immunol *20(3)*: 195-202, 2000.

36 Oka Y, Tsuboi A, Fujiki F, Li Z, Nakajima H, Hosen N, Shirakata T, Nishida S, Oji Y, Kawase I and Sugiyama H: Wt1 peptide vaccine as a paradigm for "cancer antigen-derived peptide"-based immunotherapy for malignancies: Successful induction of anti-cancer effect by vaccination with a single kind of wt1 peptide. Anti-cancer Agents Med Chem *9(7)*: 787-797, 2009.

37 Oka Y, Udaka K, Tsuboi A, Elisseeva OA, Ogawa H, Aozasa K, Kishimoto T and Sugiyama H: Cancer immunotherapy targeting wilms' tumor gene wt1 product. J Immunoly *164(4)*: 1873-1880, 2000.

38 Keilholz U, Letsch A, Busse A, Asemissen AM, Bauer S, Blau IW, Hofmann WK, Uharek L, Thiel E and Scheibenbogen C: A clinical and immunologic phase 2 trial of wilms tumor gene product 1 (wt1) peptide vaccination in patients with aml and mds. Blood *113(26)*: 6541-6548, 2009.

39 Maslak PG, Dao T, Krug LM, Chanel S, Korontsvit T, Zakhaleva V, Zhang R, Wolchok JD, Yuan J, Pinilla-Ibarz J, Berman E, Weiss M, Jurcic J, Frattini MG and Scheinberg Da: Vaccination with synthetic analog peptides derived from wt1 oncoprotein induces t-cell responses in patients with complete remission from acute myeloid leukemia. Blood *116(2)*: 171-179, 2010.

40 Tsuboi A, Oka Y, Kyo T, Katayama Y, Elisseeva OA, Kawakami M, Nishida S, Morimoto S, Murao A, Nakajima H, Hosen N, Oji Y and Sugiyama H: Long-term wt1 peptide vaccination for patients with acute myeloid leukemia with minimal residual disease. Leukemia *26(6)*: 1410-1413, 2012.

41 Rein LaM and Chao NJ: Wt1 vaccination in acute myeloid leukemia: New methods of implementing adoptive immunotherapy. Expert Opin Inv Drug *23(3)*: 417-426, 2014.

42 Sawada A, Inoue M, Kondo O, Yamada-Nakata K, Ishihara T, Kuwae Y, Nishikawa M, Ammori Y, Tsuboi A, Oji Y, Koyama-Sato M, Oka Y, Yasui M, Sugiyama H and Kawa K: Feasibility of cancer immunotherapy with wt1 peptide vaccination for solid and hematological malignancies in children. Pediatr Blood Cancer *63(2)*: 234-241, 2016.

43 Brayer J, Lancet JE, Powers J, List A, Balducci L, Komrokji R and Pinilla-Ibarz J: Wt1 vaccination in aml and mds: A pilot trial with synthetic analog peptides. Am J Hematol *90(7)*: 602-607, 2015.

44 Guo Y, Niiya H, Azuma T, Uchida N, Yakushijin Y, Sakai I and Hato T: Brief report direct recognition and lysis of leukemia cells by wt1-specific cd4 + t lymphocytes in an hla class ii – restricted manner. Cancer Res *106(4)*: 1415-1418, 2005.

45 Gaiger A, Reese V, Ml D and Ma C: Immunity to wt1 in the animal model and in patients with acute myeloid leukemia. Blood *96(4)*: 1480-1489, 2000.

46 Elisseeva OA, Oka Y, Tsuboi A, Ogata K, Wu F, Kim EH, Soma T, Tamaki H, Kawakami M, Oji Y, Hosen N, Kubota T, Nakagawa M, Yamagami T, Hiraoka A, Tsukaguchi M, Udaka K, Ogawa H, Kishimoto T, Nomura T and Sugiyama H: Humoral immune responses against wilms tumor gene wt1 product in patients with hematopoietic malignancies. Blood *99(9)*: 3272-3279, 2002.

47 Qi XW, Zhang F, Wu H, Liu JL, Zong BG, Xu C and Jiang J: Wilms' tumor 1 (wt1) expression and prognosis in solid cancer patients: A systematic review and meta-analysis. Sci Rep 5(9), 2015.

48 Tsuboi A, Oka Y, Osaki T, Kumagai T, Tachibana I, Hayashi S, Murakami M, Nakajima H, Elisseeva OA, Fei W, Masuda T, Yasukawa M, Oji Y, Kawakami M, Hosen N, Ikegame K, Yoshihara S, Udaka K, Nakatsuka S, Aozasa K, Kawase I and Sugiyama H: Wt1 peptide-based immunotherapy for patients with lung cancer: Report of two cases. Microbiol Immunol *48(3)*: 175-184, 2004.

49 Dudley ME and Rosenberg SA: Adoptive-cell-transfer therapy for the treatment of patients with cancer. Nat Rev Cancer *3(9)*: 666-675, 2003.

50 Bellantuono I, Gao L, Parry S, Marley S, Dazzi F, Apperley J, Goldman JM and Stauss HJ: Two distinct hla-a0201-presented epitopes of the wilms tumor antigen 1 can function as targets for leukemia-reactive ctl. Blood *100(10)*: 3835-3837, 2002.

51 Ohminami H, Yasukawa M and Fujita S: Hla class i-restricted lysis of leukemia cells by a cd8(+) cytotoxic t-lymphocyte clone specific for wt1 peptide. Blood *95(1)*: 286-293, 2000.

52 Gao L, Bellantuono I, Elsässer A, Marley SB, Gordon MY, Goldman JM and Stauss HJ: Selective elimination of leukemic cd34(+) progenitor cells by cytotoxic t lymphocytes specific for wt1. Blood *95(7)*: 2198-2203, 2000.

53 Sette A, Vitiello A, Reherman B, Fowler P, Nayersina R, Kast WM, Melief CJ, Oseroff C, Yuan L, Ruppert J, Sidney J, del Guercio MF, Southwood S, Kubo RT, Chesnut RW, Grey HM and Chisari FV: The relationship between class i binding affinity and immunogenicity of potential cytotoxic t cell epitopes. J Immunol *153(12)*: 5586-5592, 1994.

54 Tsuboi A, Oka Y, Udaka K, Murakami M, Masuda T, Nakano A, Nakajima H, Yasukawa M, Hiraki A, Oji Y, Kawakami M, Hosen N, Fujioka T, Wu F, Taniguchi Y, Nishida S, Asada M, Ogawa H, Kawase I and Sugiyama H: Enhanced induction of human wt1-specific cytotoxic t lymphocytes with a 9-mer wt1 peptide modified at hla-a*2402-binding residues. Cancer Immunol Immunother *51(11-12)*: 614-620, 2002.

55 Pinilla-Ibarz J, May RJ, Korontsvit T, Gomez M, Kappel B, Zakhaleva V, Zhang RH and Scheinberg Da: Improved human t-cell responses against synthetic hla-0201 analog peptides derived from the wt1 oncoprotein. Leukemia *20(11)*: 2025-2033, 2006.

56 Gajewski TF, Schreiber H and Fu Y-X: Innate and adaptive immune cells in the tumor microenvironment. Nat Immunol *14(10)*: 1014-1022, 2013.

57 Ragnarsson G NH, Chaney C, Ho B, Greenberg P: Adoptive t cell therapy targeting wt1 in leukemia and mds patients. J Immunol *182(1)*: 41-34, 2009.

58 Kim YJ, Cho SG, Lee S, Kim MS, Kim EK, Cho BS, Sohn HJ, Choi HB, Eom KS, Min CK, Kim HJ, Kim YG, Kim DW, Lee JW, Min WS, Kim CC and Kim TG: Potential role of adoptively transferred allogeneic wt1-specific cd4+ and cd8+ t lymphocytes for the sustained remission of refractory aml. Bone Marrow Transplant *45(3)*: 597-599, 2010.

59 Ochi T, Fujiwara H, Okamoto S, An J, Nagai K, Shirakata T, Mineno J, Kuzushima K, Shiku H and Yasukawa M: Novel adoptive t-cell immunotherapy using a wt1-specific tcr vector encoding silencers for endogenous tcrs shows marked antileukemia reactivity and safety. Blood *118(6)*: 1495-1503, 2011.

60 Asai H, Fujiwara H, Kitazawa S, Kobayashi N, Ochi T, Miyazaki Y, Ochi F, Akatsuka Y, Okamoto S, Mineno J, Kuzushima K, Ikeda H, Shiku H and Yasukawa M: Adoptive transfer of genetically engineered wt1-specific cytotoxic t lymphocytes does not induce renal injury. J Hematol Oncol *7(1)*: 3-3, 2014.

61 Fujiwara H, Ochi T, Ochi F, Miyazaki Y, Asai H, Narita M, Okamoto S, Mineno J, Kuzushima K, Shiku H and Yasukawa M: Antileukemia multifunctionality of cd4+ t cells genetically engineered by hla class i-restricted and wt1-specific t-cell receptor gene transfer. Leukemia April): 1-9, 2015.

62 Isao T, Masuya M, Kageyama S, Nishida T, Terakura S, Murata M, Fujiwara H, Akatsuka Y and Hiroaki Ikeda Y: Adoptive transfer of wt1-specific tcr gene-transduced lymphocytes in patients with myelodysplastic syndrome and acute myeloid leukemia. Blood *126(23)*: 97-97, 2015.

63 Yamahira A, Narita M, Ishii K, Jayathilake RMC, Iwabuchi M, Satoh N, Uchiyama T, Taniguchi T, Hashimoto S, Kasahara N, Faure E, Bogan B, Takizawa J, Sone H and Takahashi M: Enhancement of antigen presenting ability in the leukemic plasmacytoid dendritic cell line (pmdc05) by lentiviral vector-mediated transduction of cd80 gene. Leukemia Res *36(12)*: 1541-1546, 2012.

64 Yamahira A, Narita M, Nakamura T, Watanabe N, Kaji M, Taniguchi T, Hashimoto S, Furukawa T, Toba K, Aizawa Y, Kuzushima K and Takahashi M: Generation of antigen-specific cytotoxic t lymphocytes using a leukemic plasmacytoid dendritic cell line as antigen presenting cells. Leukemia Res *35(6)*: 793-799, 2011.

65 Van Tendeloo VF, Van de Velde A, Van Driessche A, Cools N, Anguille S, Ladell K, Gostick E, Vermeulen K, Pieters K, Nijs G, Stein B, Smits EL, Schroyens Wa, Gadisseur AP, Vrelust I, Jorens PG, Goossens H, de Vries IJ, Price Da, Oji Y, Oka Y, Sugiyama H and Berneman ZN: Induction of complete and molecular remissions in acute myeloid leukemia by wilms' tumor 1 antigen-targeted dendritic cell vaccination. Proc Natl Acad Sci USA *107(31)*: 13824-13829, 2010.

66 Sundarasetty BS, Singh VK, Salguero G, Geffers R, Rickmann M, Macke L, Borchers S, Figueiredo C, Schambach A, Gullberg U, Provasi E, Bonini C, Ganser A, Woelfel T and Stripecke R: Lentivirus-induced dendritic cells for immunization against high-risk wt1(+) acute myeloid leukemia. Hum Gene Ther *24(2)*: 220-237, 2013.

67 Pincha M, Sundarasetty BS, Salguero G, Gutzmer R, Garritsen H, Macke L, Schneider A, Lenz D, Figueiredo C, Blasczyk R, Ruggiero E, Schmidt M, von Kalle C, Puff C, Modlich U, von der Leyen H, Wicke DC, Ganser A and Stripecke R: Identity, potency, *in vivo* viability, and scaling up production of lentiviral vector-induced dendritic cells for melanoma immunotherapy. Hum Gene Ther *23(1)*: 38-55, 2012.

68 Salguero G, Sundarasetty BS, Borchers S, Wedekind D, Eiz-Vesper B, Velaga S, Jirmo AC, Behrens G, Warnecke G, Knöfel A-K, Blasczyk R, Mischak-Weissinger E, Ganser A and Stripecke R: Preconditioning therapy with lentiviral vector-programmed dendritic cells accelerates the homeostatic expansion of antigen-reactive human t cells in nod.Rag1-/-.Il-2grganmac-/- mice. Hum Gene Ther *22(10)*: 1209-1224, 2011.

69 Willemen Y, Van den Bergh JMJ, Lion E, Anguille S, Roelandts VAE, Van Acker HH, Heynderickx SDI, Stein BMH, Peeters M, Figdor CG, Van Tendeloo VFI, de Vries IJ, Adema GJ, Berneman ZN and Smits ELJ: Engineering monocyte-derived dendritic cells to secrete interferon-α enhances their ability to promote adaptive and innate anti-tumor immune effector functions. Cancer Immunol Immunother *64(7)*: 831-842, 2015.

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