

“Don’t Eat Me” Signals of Neuroblastoma by CD47 for Immune Escape: A Novel Prognostic Biomarker [†]

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Abstract: Recent studies have shown that cancer cells can deceive phagocytosing macrophage cells through the CD47 protein which gives the message “don’t eat me” or “don’t kill me” to immune components. The efficacy of anti-CD47 treatment approach was shown in cancers such as, non-small cell lung cancer, non-Hodgkin lymphoma, ovarian cancer, and breast cancer. The studies on the immunobiology of neuroblastoma has increased as monoclonal antibody based immunotherapy has shown to be effective in high-risk patients such as anti disialoganglioside. Therefore, the aim of this study was to evaluate the levels of CD47 protein expression among neuroblastoma patients with different risk groups and genetic alterations. This study included paraffin-embedded tumor tissues of 66 neuroblastoma patients (28 girls, 38 boys) with an age range of 0.5 to 108 months with a mean value of 24.9 (± 23.5). According to risk classifications 21 were at low risk (31.8%), 24 were at intermediate risk (36.4%) and 19 were at high-risk (28.8%) groups. These samples were evaluated for MYCN amplification, 1p36 LOH, 11q23 deletion and 17q25 gain by real-time PCR. In addition, CD47 expression status (positive or negative) was detected by immunohistochemical analysis. All data was analyzed with Chi-Square and Mann-Whitney U non-parametric tests within SPSS program, version 22.0. *p*-value lower than 0.5 was found statistically significant. According to the results, patients at low risk did not express CD47, while patients at high-risk group were mostly expressing CD47 (*p* = 0.049). MYCN amplification positive patients were expressing CD47 protein (*p* = 0.046). Patients without 17q25 gain were found to be expressing CD47 protein (*p* = 0.006). In addition, CD47 expression was increasing as age was getting higher in terms of months (*p* = 0.018). The findings of this study suggest that positive expression pattern of CD47 may be a poor prognostic biomarker especially in high risk 17q gain negative neuroblastoma patients.

Keywords: CD47; neuroblastoma; Cancer Cell Death Escape; survivin

1. Introduction

Immuno-oncology research is conducted on the mechanisms by which the tumor cells evade immune-surveillance. The essential approach of immune therapy is to reactivate the immune system to eliminate tumor cells. Immunotherapy drugs such as nivolumab, pembrolizumab, atezolizumab and ipilimumab were developed against PD-1, PD-L1 and CTLA-4 proteins, which have enabled T cells to achieve successful results in cancer treatment. Today, they are licensed and used in the treatment of some cancer types [1,2].

Recent studies have shown that cancer cells can deceive phagocytosing macrophage cells through the CD47 protein. CD47, in other terms integrin-associated protein (IAP) is a transmembrane glycoprotein which gives the message “don’t eat me” or “don’t kill me” to immune components. In this way, macrophage cells perceive tumor cells as normal healthy cells of the host. Cancer stem cells also survive and resist by secreting CD47 protein [3,4]. According to some preclinical studies on targeting CD47, its efficacy was shown in cancers such as, non-small cell lung cancer, non- Hodgkin lymphoma, ovarian cancer, breast cancer that lead to clinical trials [5,6]. Overexpression pattern of this protein was associated with poor prognosis in leukemia [7]. In addition, this glycoprotein also plays crucial roles in both healthy and pathological cases including autoimmunity, red blood cell homeostasis, infection, inflammation [8].

Neuroblastoma is the most common extracranial solid localized tumor originating from the primordial neural crest cells normally found in adrenal medulla or sympathetic ganglia and constituting 8% to 10% of all childhood cancers. It is responsible for 15% of cancer-related deaths in this age group [9]. The effect of many cytogenetic and molecular genetic changes in tumor tissue on prognosis was shown in neuroblastoma. For example; While “near triploidy” is associated with good prognosis, MYCN oncogene amplification and diploidy indicates poor prognosis. In addition, unbalanced translocation of 17q (17q gain), allelic loss of 1p or 11q are associated with more aggressive tumor characteristics and poor prognosis [10,11]. The studies on the immunobiology of neuroblastoma has increased as monoclonal antibody based immunotherapy has shown to be effective in high-risk patients such as anti disialoganglioside (GD2). Therefore, new immunotherapeutic approaches should be developed to reveal new targets in neuroblastoma especially in high risk or minimal residual disease conditions [11].

There is no previous study evaluating the expression pattern of CD47 in neuroblastoma in the literature. The aim of this study was to evaluate the levels of CD47 protein expression among neuroblastoma patients with different risk groups and genetic alterations.

2. Material and Methods

2.1. Patients and Sample Collection

Paraffin embedded tumor tissues of 66 neuroblastoma patients were collected within the “Neuroblastoma 2009 Protocol of Turkish Pediatric Oncology Group”. Informed consent form was signed to patient’s caregivers and parents within this protocol. The information of age (months), gender, histopathological diagnosis, histological differentiation and risk group were collected in web-based database of the protocol.

2.2. DNA Isolation and Real-Time PCR Analysis

After de-paraffinization of tumor samples, DNA samples were isolated according to the manufacturer’s protocol. After adding binding buffer and proteinase K, cell membrane was lysed. Isopropanol was applied on samples followed by consecutive washing steps. The concentrations of the isolated DNA samples were measured by spectrophotometer and these samples were evaluated for MYCN amplification, 1p36 LOH, 11q23 deletion and 17q25 gain by real-time PCR. In order to determine the gains or losses in these regions, primer and enzyme mixtures containing labeled probes specific to these regions were used. According to Cq values, more than 10 fold expression of MYCN was considered as amplification positive, less than 0.5 fold 1p36 and 11q23 expressions were considered as LOH and deletion positive while more than 1.1 delta Cq value of 17q25 was evaluated as gain positive.

2.3. Immunohistochemical Analysis of CD47

Automatic staining with Ventana Discovery device was performed for the immunohistochemical analysis of CD47 expression levels. The tumor tissue paraffin-sections were taken on positively charged slides of 3-micron diameter. After overnight incubation at 60 degrees, the deparaffinization and preparation steps were carried out by loading to Ventana Discovery device. Then, primary

antibody was applied with blocking at 1/200 dilution. After incubating for an hour, DAB chromogen and streptavidin biotin peroxidation stainings were performed. After this process, hematoxylin staining was performed and transparent slides were closed with entellan. The CD47 expression pattern was recorded according to grading (+: low positive, ++: moderately positive, +++: highly positive) and percentage levels by examining under inverted light microscope. The final scoring was carried out with a combined assessment approach of both, resulting as +: CD47 positive or -: CD47 negative.

2.4. Statistical Analysis

All data were analyzed by SPSS program, version 22.0. All data obtained were categorical variables (except age), thus statistical analysis were performed according to Chi Square Test. The statistical tests for evaluation of age and other variables were selected based on normal distribution within groups. *p*-values less than 0.05 were considered statistically significant.

3. Results

3.1. Patient Characteristics

This study included 66 patients with neuroblastoma. Age range was between 0.50 to 108 months with a mean value of 24.9 (± 23.5). Twenty-eight were girls (42.4%) and 38 were boys (57.6%). According to Shimada histology 46 had favorable histology (69.7%), while 20 had unfavorable histology (30.3%). The risk classifications were as follows; 21 were at low risk (31.8%), 24 were at intermediate risk (36.4%) and 19 were at high-risk (28.8%) groups.

3.2. RT-PCR Analysis Results

According to MYCN amplification, 1p36 LOH, 11q23 deletion and 17q25 gain analysis, the distribution of all cases were as follows: 18 were MYCN amplification positive (27.3%), 33 were 1pLOH positive (50%), 9 were 11q23 deletion positive (13.6%) and 35 were 17q25 gain positive (53.0%).

3.3. CD47 Expression Final Scores According to Immunohistochemical Analysis

According to evaluation of grading and expression percentages 28 of all cases were CD47 positive (42.4%) while 38 were negative (57.6%). In low risk group ($n = 21$), there were nine cases with CD47 positive expression pattern (42.9%); among 24 cases at intermediate risk group 14 were CD47 positive (58.3%) and 14 of 19 cases at high-risk group, were CD47 positive (73.7%).

3.4. Statistical Analysis Results

According to Pearson-Chi Square Test, CD47 expression did not show statistically significant relation among three risk groups ($p = 0.143$). However, when this evaluation was performed between high and low risk patients, there were a statistically significant relation ($p = 0.049$, Chi Square–Fisher’s Exact test). This relation indicated that patients at low risk did not express CD47, while patients at high-risk group were mostly expressing CD47. There was statistically significant relation between MYCN amplification status and CD47 expression ($p = 0.046$, Chi Square–Fisher’s Exact test). MYCN amplification positive patients were expressing CD47 protein. 1p36 LOH and 11q23 deletion status did not show statistically significant relation with CD47 expression ($p = 0.539$ and $p = 0.287$, respectively). However, patients without 17q25 gain were found to be expressing CD47 protein ($p = 0.006$). Gender of patients was not in statistically significant relation with CD47 expression ($p = 0.344$ Chi Square–Fisher’s Exact test) while age was a statistically significant factor for CD47 expression ($p = 0.018$, Mann-Whitney U non-parametric test). Mean age of patients with positive CD47 pattern (30.48 ± 25.01 months) were higher than patients with negative CD47 pattern (14.75 ± 12.37 months), indicating that CD47 expression was increasing as age was getting higher in terms of months.

4. Discussion

This study was planned to evaluate the distribution of CD47 expression patterns among 66 neuroblastoma patients at different risk groups with various genetic alterations. The study supported our hypothesis that CD47 expression varies among risk groups in neuroblastoma with our finding that low risk neuroblastoma patients did not express CD47 protein.

The role of CD47 expression is not well identified in pediatric cancers. However it was shown that anti-CD47 antibody had a synergistic effect with rituximab by promoting phagocytosis in non-Hodgkin lymphoma [12]. According to Ridler and his colleagues (2017) review of preclinical experimental animal studies on treatment with antibody against CD47, increased number of macrophages in medulloblastoma and high-grade glioma were found. In addition, this treatment (Hu5F9-G4) did not lead to phagocytosis of normal brain cells, which indicates that anti-CD47 therapy can be promising with its immune inducing and safe effects in neuronal originated cancers [13]. Tan and his colleagues (2015) showed that antigens of CD47 may play crucial roles in the process of ovarian cancer development as a metastasis promoting risk factor and therefore may constitute an immunotherapeutic target [14]. Our finding of negative expression pattern in low risk neuroblastoma was found to be in correlation with the literature.

CD47 expression pattern was found to be positive in MYCN amplification positive patients, which was thought to be associated with prognosis and risk classification. MYCN amplification is known to be a poor prognostic biomarker and increasingly amplified in high-risk neuroblastoma patients [10]. We found that CD47 expression was negative among low risk neuroblastoma patients. CD47 positiveness was also increasing with age, which is known to be poor prognostic factor in neuroblastoma [10]. Therefore, these all findings suggest that negative expression pattern of CD47 may be a good predictive biomarker of neuroblastoma.

Additionally, the relation between 17q25 gain negativeness and CD47 positiveness was a remarkable finding for understanding neuroblastoma cells' death escape. 17q25 gene encodes survivin which is a crucial anti-apoptotic protein, highly expressed in most cancers [15]. Gain of 17q25 results in evade of neuroblastoma cells from apoptosis. However, our results showed that CD47 expression was positive in patients with negative 17q25 gain status. In this case, positive expression pattern of CD47 which is located in 3q13, may present an alternative way of evading apoptosis by immune escape from macrophagic phagocytosis.

5. Conclusions

The findings of this study suggest that positive expression pattern of CD47 may be a poor prognostic biomarker especially in high risk 17q gain negative neuroblastoma patients. We assert that the power gained by neuroblastoma cells to evade cell death in 17q25 gain negative cases might be due to the expression of CD47, which is not defined in the literature yet. In further studies, it is aimed to evaluate the 3q13 locus encoding CD47 in detail to elucidate this escape mechanism.

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