Research Article

Expression of FOXP3, CD14, and ARG1 in Neuroblastoma Tumor Tissue from High-Risk Patients Predicts Event-Free and Overall Survival

Sara Stigliani, Michela Croce, Fabio Morandi, Paola Scaruffi, Valentina Rigo, Barbara Carlini, Carla Manzetti, Anna Rita Gigliotti, Gian Paolo Tonini, Vito Pistoia, Silvano Ferrini, and Maria Valeria Corrias

1Physiopathology of Human Reproduction, IRCCS A.O.U. San Martino-IST, 16132 Genoa, Italy
2Laboratory of Biotherapy, IRCCS A.O.U. San Martino-IST, 16132 Genoa, Italy
3Laboratory of Oncology, IRCCS Istituto Giannina Gaslini, 16148 Genoa, Italy
4Oncology Unit, IRCCS Istituto Giannina Gaslini, 16148 Genoa, Italy
5Epidemiology, Biostatistics and Committees Unit, IRCCS Istituto Giannina Gaslini, 16148 Genoa, Italy
6Neuroblastoma Laboratory, Pediatric Research Institute, Fondazione Città della Speranza, 35127 Padua, Italy

Correspondence should be addressed to Maria Valeria Corrias; mariavaleriacorrias@ospedale-gaslini.ge.it

Received 10 July 2014; Revised 12 January 2015; Accepted 14 January 2015

Academic Editor: Mohammad Owais

Copyright © 2015 Sara Stigliani et al. This is an open access article distributed under the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

The prognosis of children with metastatic neuroblastoma (NB) > 18 months at diagnosis is dismal. Since the immune status of the tumor microenvironment could play a role in the history of disease, we evaluated the expression of CD45, CD14, ARG1, CD163, CD4, FOXP3, Perforin-1 (PRF1), Granzyme B (GRMB), and IL-10 mRNAs in primary tumors at diagnosis from children with metastatic NB and tested whether the transcript levels are significantly associated to event-free and overall survival (EFS and OS, resp.). Children with high expression of CD14, ARG1 and FOXP3 mRNA in their primary tumors had significantly better EFS. Elevated expression of CD14 and FOXP3 mRNA was significantly associated to better OS. CD14 mRNA expression levels significantly correlated to all markers, with the exception of CD4. Strong positive correlations were found between PRF1 and CD163, as well as between PRF1 and FOXP3. It is worth noting that the combination of high levels of CD14, FOXP3, and ARG1 mRNAs identified a small group of patients with excellent EFS and OS, whereas low levels of CD14 were sufficient to identify patients with dismal survival. Thus, the immune status of the primary tumors of high-risk NB patients may influence the natural history of this pediatric cancer.

1. Introduction

Neuroblastoma (NB) is a pediatric neuroectodermal solid tumor with a heterogeneous clinical behavior [1]. Despite intensive multimodal therapy, patients presenting with metastatic disease, that is, stage 4 according to INSS [2] or stage M according to INRG-SS [3], aged more than 18 months at diagnosis have dismal survival rate. The search for powerful prognostic markers for this subset of patients is aimed to identify the cases that can be cured by standard therapy and the ultra-high-risk cases that need to be enrolled in new experimental trials.

Recently, the presence of high levels of NB-related molecular markers in bone marrow (BM) and peripheral blood (PB) samples at diagnosis has been shown to be highly predictive of event free survival (EFS) and overall survival (OS) [4]. However, a prognostic marker in the primary tumor could be helpful to improve patients’ stratification. Unfortunately, although several gene expressions profiling studies of primary tumor specimens have identified prognostic signatures [5–12], so far none of the latter has a predictive power within the subset of stage 4 patients aged > 18 months at diagnosis.

It is increasingly evident that the tumor microenvironment plays an important role in driving the fate of antitumor
response (see [13] for a review). Indeed, several membrane-bound or soluble factors produced by normal and neoplastic cells in the tumor microenvironment may downregulate the antitumor immune response and greatly influence the natural history of cancer. Recently, specific gene signatures related to a successful immune response and to tumor rejection processes have been related to tumor outcome in different tumors [14].

In human NB, information on the presence and activity of specific subsets of immune suppressive cells and soluble factors in the primary tumors is scanty [15]. Facchetti et al. [16] showed that NB primary tumors show different degrees of lymphocyte infiltration, but no correlation with survival was found. A gene expression study performed on primary tumors [12] suggested a negative role for myeloid-derived suppressor cells in the prognosis of metastatic NB patients. Recently, the same authors [17, 18] demonstrated that the inclusion of 5 inflammation related genes increased the predictive power of the gene signature, since tumors from high-risk NB patients present a greater infiltration of CD163+, M2-type, tumor associated macrophages (TAMs). Interestingly, NKT cells can be instructed to selectively kill these TAMs [18].

An important immune suppressive role is ascribed to CD4+CD25highFoxP3+ T cells, also termed Treg cells. No difference in their number was found in PB samples from a small cohort of low- and high-risk patients [19]. However, Tilak et al. have recently shown that the frequency of Treg in PB samples was higher in NB patients than in healthy children and that frequency was reduced after chemotherapy [20]. Nevertheless, the analysis of Treg in several tumor types indicated that FoxP3+ expression may be related to CD4+ T-cell activation rather than to an immune suppressive phenotype [21, 22]. This finding has important clinical implications since depletion of CD4+CD25highFoxP3+ cells has been proposed as a tool to enhance tumor responses. Indeed, Carlson and coworkers [23] recently demonstrated that NB tumor-infiltrating CD4+ T cells can be activated in the tumor milieu, but not in the periphery. Gowda et al. [19] surprisingly found that the immunosuppressive cytokine IL-10, produced by Treg, T regulatory type 1 (Tr1) cells [24], and cells of the innate immunity, such as NK and macrophages, was elevated in PB of patients with low-risk NB, suggesting a protective role of innate immunity.

To gain insight into the role of different immune cell populations in the natural history of metastatic NB, we have evaluated the mRNA expression of the following molecular markers in 41 primary tumors at diagnosis: CD45: all leukocytes, CD4: monocyte-macrophages, ARG1: activated macrophages, CD163: M2 TAM, CD4: T helper cells, FOXP3: Treg, Perforin-1 (PRF1), and Granzyme B (GRMB): cytotoxic T lymphocytes and activated NK cells. In addition we evaluated the mRNA expression of the immune suppressive cytokine IL-10. We then tested whether expression of these genes significantly correlated to survival.

| Table I: Patients’ characteristics. |
|-------------------------------------|
| Age at diagnosis | N | % |
| <18 months | 0 | |
| >18 months | 41 | 100 |
| Sex | | |
| Female | 17 | 41.5 |
| Male | 24 | 58.5 |
| MYCN status | | |
| Amplified | 13 | 31.7 |
| Not amplified | 28 | 68.3 |
| Primary tumor site | | |
| Adrenal | 25 | 61.0 |
| Thorax | 3 | 7.3 |
| Abdomen | 13 | 31.7 |
| Metastatic sites | | |
| Bone Marrow | 13 | 31.7 |
| Bone Marrow + bone | 24 | 58.6 |
| Pleura | 2 | 4.9 |
| Bone | 1 | 2.4 |
| Other | 1 | 2.4 |
| Relapse | | |
| No | 15 | 36.6 |
| Yes | 26 | 63.4 |
| Outcome | | |
| Alive | 16 | 39.0 |
| Dead of disease | 25 | 61.0 |

2. Materials and Methods

2.1. Patients and Tumors. Patients included in the study were diagnosed in Italy with stage 4 NB between December 1992 and October 2006. Disease staging [2] was made at the referring oncology center and centrally reviewed at the Gaslini Institute. The median age at diagnosis was 3.4 years (range 1.5–6.3) and the median follow-up was 46.7 months (range 0.3–172.4). Patients were treated according to protocols NB-92, NB-95, and NB-97, which include induction therapy followed by myeloablative chemotherapy with autologous stem cell transplantation for consolidation. Survival rates for these protocols have been demonstrated to be similar [25] and are in line with the expected survival rate for patients with metastatic NB aged > 18 months at diagnosis [3]. Follow-up data at January 2014 were retrieved from the Italian Neuroblastoma Registry (INBR) [25]. Patients’ characteristics are reported in Table I.

After histological diagnosis, an aliquot of the primary tumor surgically resected at diagnosis was centralized at the Gaslini Institute and stored at −80°C until RNA was extracted. Only tumors with neoplastic cell content higher than 80% were included.
The study was approved by the Institutions' Ethical Committees and all analyses were performed according to the Helsinki declaration.

2.2. RNA Extraction and RT-qPCR Analysis. Total RNA was extracted from primary tumors as previously described [26]. One hundred ng of total RNA was reverse transcribed and then amplified for each molecular marker in duplicate by qPCR, using the following assays from Life Technology (Life Technologies Europe BV, Monza, Italy): CD45: Hs00365634_g1, CD14: Hs02621496_s1, CD163: Hs00174705_m1, ARG1: Hs00968979_m1, CD4: Hs01058407_m1, FOXP3: Hs00203958_m1, IL10: Hs00961622_m1, PRF1: Hs00169473_m1, GZMB: Hs01554355_m1, and primers and probes for β2-microglobulin (B2M) [27]. The level of expression of each marker was normalized to the expression of B2M, according to the delta Ct method [28] and results were reported as 2^-delta Ct. All markers were tested on a panel of 10 NB cell lines and none of these markers was expressed by the tumor cells themselves. Moreover, to exclude DNA contamination the cDNA obtained in the absence of reverse transcriptase was included in each qPCR assay. Water was also run as negative control.

2.3. Statistical Analysis. The Wilcoxon-Mann-Whitney test was used to compare median values, and the Spearman ρ coefficient was used to assess correlation between variables. Event-free and overall survival (EFS and OS, resp.) analyses were performed according to the Kaplan-Meier method and compared by the log-rank test. A P value < 0.05 was considered as statistically significant. Analyses were made using the Prism software (GraphPad Software Inc., La Jolla, CA).

3. Results

3.1. Expression of Molecular Markers for Different Immune Cell Populations in Primary NB Tumors. We evaluated the expression of CD45, CD14, CD163, ARG1, CD4, FOXP3, PRF1, GZMB, and IL10 mRNAs in primary tumor samples taken at diagnosis from 41 patients with metastatic NB >18 months at diagnosis. After normalization to B2M expression, the median expression value was used to stratify patients and correlation with EFS and OS was tested by the log rank test. High-risk patients with high expression of CD14, ARG1, and FOXP3 mRNA in their primary tumors had a significantly better EFS (Figures 1(b), 1(d), and 1(f), P = 0.0083, P = 0.0482, and P = 0.0024, resp.). In addition, a trend toward a better survival was seen for patients with high expression of CD45 and PRF1 (Figures 1(a) and 1(g), resp.). The OS was significantly better for patients with high CD14 and FOXP3 RNA expression in their primary tumors at diagnosis (Figures 2(b) and 2(f), P = 0.0008 and P = 0.0022, resp.). It is worth noting that all the patients with CD14 expression below the median died of disease. Overall survival was also better for patients that had high CD45 and PRF1 expression (Figures 2(a) and 2(g), P = 0.0266 and P = 0.0486, resp.).

Levels of CD163, CD4, GZMB, and IL10 mRNA in primary tumors of stage 4 NB patients were never associated to different EFS or OS (Figures 1 and 2, resp.).

3.2. Correlation of Expression Levels of Molecular Markers. We then analyzed potential correlation in the expression of the molecular markers. CD14 mRNA expression levels significantly correlated to all the other markers (Figures 3(a) to 3(f)), with the exception of CD4 (not shown). Surprisingly, strong positive correlations were found between PRF1 and CD163, as well as between PRF1 and FOXP3 (Figures 3(g) and 3(h), resp.). Positive correlations were also found between IL10 and ARG1, between IL10 and CD4 (Figures 3(j) and 3(k), resp.), and between CD163 and ARG1 (Figure 3(l)).

3.3. Predictive Power of Molecular Markers. Based on the results of survival analyses and correlation studies, we tested whether the combination of markers had a higher predictive power than a single marker. As shown in Figure 4, the combination of high levels of CD14, FOXP3, and ARG1 expression strongly predicted good EFS and OS. However, low levels of CD14 remained the best predictor of a dismal survival (Figure 2(b)).

3.4. Analysis of a Public NB Tumor Gene Expression Profiling Dataset. We checked in a public gene expression profiling dataset of 40 stage 4 NB tumors (R2: Genomics Analysis and Visualization Platform: http://hgservrl.amc.nl/cgi-bin/r2/main.cgi, tumor neuroblastoma, Versteeg) significant associations between the expression levels of the studied molecular markers and EFS and OS. It is important to note that the public dataset reports microarray data and that Kaplan-Meyer plot is given for each probe according to ROC analysis. Despite the difference in the type of data and analysis, the significant association of high FOXP3 mRNA levels with better EFS and OS was confirmed (Figure 5). No significant association was found for CD14 and ARG1 (not shown).

4. Discussion

The prognostic role of molecular analysis of immune cells and of the immunosuppressive cytokine IL10 has been evaluated in primary tumors from 41 children with metastatic NB aged more than 18 months at diagnosis. The results indicate that high level of CD14, ARG1, and FOXP3 mRNA expression in primary NB tumor significantly correlated to a better survival. Each of these markers had predictive power; however, the combined use of the three markers improved survival prediction and allowed to identify patients that can be cured by standard therapy. Furthermore, the association of FOXP3 mRNA levels with different EFS and OS was confirmed in a gene expression dataset of 40 stage 4 NB tumors analyzed by microarray. The discrepancy in the predictive power of CD14 and ARG1 observed by RT-qPCR and microarray analysis may relate to the different sensitivity/specificity of the oligonucleotide probes used. Moreover, in the Versteeg’s database the tumor cell content required for inclusion was...
lower and patients < 18 months could not be excluded. Thus, our findings need to be confirmed by qPCR in a prospective study with a greater cohort of stage 4 NB patients > 18 months.

The demonstration that FOXP3 expression positively associated to a better EFS and OS of high-risk NB patients is in agreement with previous reports in head and neck and colorectal cancer patients [29, 30]. It has been suggested that activation state or suppressive functions of the infiltrating T cells greatly depend on the specific microenvironment present in the anatomical site [21]. FOXP3 expression did not correlate to CD4 expression but correlated to PRF1 mRNA levels, further supporting the tenet that FOXP3 mRNA expression in NB tumors was an indicator of effector T-cell activation rather than of immunosuppressive Treg cells [21, 22, 31–33]. Since FOXP3 expression correlated to CD14 expression, the presence of macrophages or dendritic cells
expressing CD14 may be responsible for the activation of effector T cells, which in turn limited tumor progression. It is of note that CD14 expression levels, but not those of the TAM marker CD163, significantly associated to different survival, suggesting that CD14+ cells other than TAMs may favor the induction of an effective immune response.

Regarding the role of FoxP3-expressing cells, in murine syngeneic NB models, CD4+FoxP3+ Treg cells increase in secondary lymphoid organs of NB-bearing mice and their depletion increased the effects of immunotherapy and of hematopoietic stem cell transplantation [34–37]. The apparent discrepancy between syngeneic murine models and human NB may relate to a different expression of FoxP3 in activated effector T cells in mouse and human [33]. In addition, human NB cells express low, if any, levels of HLA class I molecules [38]. This defect may hamper their
Figure 3: Significant r Spearman's correlations between CD14 and CD45 (a), CD163 (b), ARG1 (c), FOXP3 (d), GZMB (e), and PRF1 (f); between PRF1 and CD163 (g) and FOXP3 (h); between IL10 and CD45 (i), ARG1 (j), and CD4 (k); and between CD163 and ARG1 (l).
Figure 4: Kaplan-Meier plots of EFS (left panels) and OS (right panels) of stage 4 patients stratified according to the level of mRNA expression below (continuous line) or above (dotted line) the median for FOXP3 and CD14 ((a) and (b)), CD14 and ARG1 ((c) and (d)), and for FOXP3, CD14, and ARG1 ((e) and (f)). The number of patients in each curve is given in brackets.
**Tumor neuroblastoma public-Versteeg-88**

**MAS5.0_u133p2**

**FOXP3** (221334_s_at)

Expression cut-off: 4.0 (min.grp = 8)

---

**Figure 5:** Kaplan-Meyer plots of EFS (a) and OS (b) of stage 4 patients from the public NB database stratified according to the level of **FOXP3** mRNA expression below or above the ROC determined cut-off.
recognition by CD8+ T cells but on the other hand may facilitate NK cell-mediated killing [39]. Nonetheless, HLA class I molecule expression can be restored in human NB cells by IFN-γ [40], produced by activated T and NK cells, allowing NB cell recognition by CTLs. It is important to note that NKT cells have been involved in the immune response to human NB [18] and that activated NKT cells may express FoxP3 [41]. Therefore a possible role of activated NKT cells cannot be excluded. Finally, since low expression of all tested molecular markers, although not significantly, associated to a worse survival, the possibility that all infiltrating immune cells may play a role in improving antitumor responses needs to be considered.

Taken together, our findings suggest that if the primary tumor of stage 4 NB patients >18 months is infiltrated by FoxP3-expressing effector T or NKT cells, an effective antitumor response may take place and cooperate with standard therapy to increase survival.

5. Conclusions

High expression of FOXP3, CD14, and ARG1 mRNA in the primary tumors of high-risk NB patients was predictive of better survival, suggesting that the immune status of the tumor may influence the natural history of this pediatric cancer.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Authors’ Contribution

Sara Stigliani and Michela Croce equally contributed to the work.

Acknowledgments

This study was supported by Fondazione Italiana per la Lotta al Neuroblastoma (to Maria Valeria Corrias and Michela Croce), by AIRC IGI3518 (to Silvano Ferrini), and by Ministero Della Salute (Ricerca Corrente and Cinque per mille to Vito Pistoia and Silvano Ferrini). Sara Stigliani is a recipient of AIRC fellowship, Barbara Carlini and Michela Croce are recipients of Fondazione Italiana per la lotta al neuroblastoma fellowships. The excellent data management of INRB by Giovanni Erminio is deeply acknowledged.

References

[1] N.-K. V. Cheung and M. A. Dyer, “Neuroblastoma: developmental biology, cancer genomics and immunotherapy,” Nature Reviews Cancer, vol. 13, no. 6, pp. 397–411, 2013.
[2] G. M. Brodeur, J. Pritchard, F. Berthold et al., “Revisions of the international criteria for neuroblastoma diagnosis, staging, and response to treatment,” Journal of Clinical Oncology, vol. 11, no. 8, pp. 1466–1477, 1993.
[3] S. L. Cohn, A. D. J. Pearson, W. B. London et al., “The International Neuroblastoma Risk Group (INRG) classification system: an INRG task force report,” Journal of Clinical Oncology, vol. 27, no. 2, pp. 289–297, 2009.
[4] V. F. Viprey, W. M. Gregory, M. V. Corrias et al., “Neuroblastoma mRNAs predict outcome in children with stage 4 neuroblastoma: a European HR-NBLI/SIOPEN study,” Journal of Clinical Oncology, vol. 32, no. 10, pp. 1074–1083, 2014.
[5] J. Vermeulen, K. de Preter, A. Narango et al., “Predicting outcomes for children with neuroblastoma using a multigene-expression signature: a retrospective SIOPEN/COG/GPOH study,” The Lancet Oncology, vol. 10, no. 7, pp. 663–671, 2009.
[6] J. Takita, M. Ishii, S. Tsutsumi et al., “Gene expression profiling and identification of novel prognostic marker genes in neuroblastoma,” Genes Chromosomes and Cancer, vol. 40, no. 2, pp. 120–132, 2004.
[7] A. Schramm, J. H. Schulte, L. Klein-Hitpass et al., “Prediction of clinical outcome and biological characterization of neuroblastoma by expression profiling,” Oncogene, vol. 24, no. 53, pp. 7902–7912, 2005.
[8] M. Ohira, S. Oba, Y. Nakamura et al., “Expression profiling using a tumor-specific cDNA microarray predicts the prognosis of intermediate risk neuroblastomas,” Cancer Cell, vol. 7, no. 4, pp. 337–350, 2005.
[9] A. Oberthuer, B. Hero, F. Berthold et al., “Prognostic impact of gene expression-based classification for neuroblastoma,” Journal of Clinical Oncology, vol. 28, no. 21, pp. 3506–3515, 2010.
[10] E. Hiyama, K. Hiyama, H. Yamaoka, T. Sueda, C. P. Reynolds, and T. Yokoyama, “Expression profiling of favorable and unfavorable neuroblastomas,” Pediatric Surgery International, vol. 20, no. 1, pp. 33–38, 2004.
[11] Q.-R. Chen, Y. K. Song, J. S. Wei et al., “An integrated cross-platform prognosis study on neuroblastoma patients,” Genomics, vol. 92, no. 4, pp. 195–203, 2008.
[12] S. Asgharzadeh, R. Pique-Regi, R. Sposto et al., “Prognostic significance of gene expression profiles of metastatic neuroblastomas lacking MYCN gene amplification,” Journal of the National Cancer Institute, vol. 98, no. 17, pp. 1193–1203, 2006.
[13] D. Lindau, P. Gielen, M. Kroesen, P. Wesseling, and G. J. Adema, “The immunosuppressive tumour network: myeloid-derived suppressor cells, regulatory T cells and natural killer T cells,” Immunology, vol. 138, no. 2, pp. 105–115, 2013.
[14] J. Galon, H. K. Angell, D. Bedognetti, and F. M. Marincola, “The continuum of cancer immunosurveillance: prognostic, predictive, and mechanistic signatures,” Immunity, vol. 39, no. 1, pp. 11–26, 2013.
[15] R. C. Seeger, “Immunology and immunotherapy of neuroblastoma,” Seminars in Cancer Biology, vol. 21, no. 4, pp. 229–237, 2011.
[16] P. Facchetti, I. Prigione, F. Ghio, P. Tasso, A. Garaventa, and V. Pistoia, “Functional and molecular characterization of tumour-infiltrating lymphocytes and clones thereof from a major-histocompatibility-complex-negative human tumour: neuroblastoma,” Cancer Immunology, Immunotherapy, vol. 42, no. 3, pp. 170–178, 1996.
[17] S. Asgharzadeh, J. A. Salo, L. Ji et al., “Clinical significance of tumor-associated inflammatory cells in metastatic neuroblastoma,” Journal of Clinical Oncology, vol. 30, no. 28, pp. 3525–3532, 2012.
[18] L. Song, S. Asgharzadeh, J. Salo et al., “Vα24-invariant NKT cells mediate antitumor activity via killing of tumor-associated...
macrophages,” *The Journal of Clinical Investigation*, vol. 119, no. 6, pp. 1524–1536, 2009.

[19] M. Gowda, K. Godder, M. Kmieciak et al., “Distinct signatures of the immune responses in low risk versus high risk neuroblastoma,” *Journal of Translational Medicine*, vol. 9, no. 1, article 170, 2011.

[20] T. Tilak, S. Sherawat, S. Agarwala, R. Gupta, S. Vishnubhatla, and S. Bakhshi, “Circulating t-regulatory cells in neuroblastoma: a pilot prospective study,” *Pediatric Hematology and Oncology*, vol. 31, no. 8, pp. 717–722, 2014.

[21] F. Martin, S. Ladoire, G. Mignot, L. Apetoh, and F. Ghiringhelli, “Human FOXP3 and cancer,” *Oncogene*, vol. 29, no. 29, pp. 4121–4129, 2010.

[22] R. J. DeLeeuw, S. E. Kost, J. A. Kakal, and B. H. Nelson, “The prognostic value of FoxP3+ tumor-infiltrating lymphocytes in cancer: a critical review of the literature,” *Clinical Cancer Research*, vol. 18, no. 11, pp. 3022–3029, 2012.

[23] L. M. Carlson, A. de Geer, B. Sveinbjornsson et al., “The microenvironment of human neuroblastoma supports the activation of tumor-associated T lymphocytes,” *Oncology Immunology*, vol. 2, Article ID e23618, 2013.

[24] N. Gagliani, C. F. Magnani, S. Huber et al., “Coexpression of CD49b and LAG-3 identifies human and mouse T regulatory type 1 cells,” *Nature Medicine*, vol. 19, pp. 739–746, 2013.

[25] R. Haupt, A. Garaventa, C. Gambini et al., “Improved survival of children with neuroblastoma between 1979 and 2005: a report of the Italian neuroblastoma registry,” *Journal of Clinical Oncology*, vol. 28, no. 14, pp. 2331–2338, 2010.

[26] P. Scaruffi, S. Stigliani, S. Moretti et al., “Transcribed-ultra conserved region expression is associated with outcome in high-risk neuroblastoma,” *BMC Cancer*, vol. 9, article 441, 2009.

[27] V. F. Viprey, M. V. Corrias, B. Kagedal et al., “Standardisation of operating procedures for the detection of minimal disease by QRT-PCR in children with neuroblastoma: quality assurance on behalf of SIOPEN-R-NET,” *European Journal of Cancer*, vol. 43, no. 2, pp. 341–350, 2007.

[28] K. J. Livak and T. D. Schmittgen, “Analysis of relative gene expression data using real-time quantitative PCR and the 2−ΔΔCT method,” *Methods*, vol. 25, no. 4, pp. 402–408, 2001.

[29] C. Badoual, S. Hans, J. Rodriguez et al., “Prognostic value of tumor-infiltrating CD4+ T-cell subpopulations in head and neck cancers,” *Clinical Cancer Research*, vol. 12, no. 2, pp. 465–472, 2006.

[30] P. Salama, M. Phillips, F. Grieu et al., “Tumor-infiltrating FOXP3+ T regulatory cells show strong prognostic significance in colorectal cancer,” *Journal of Clinical Investigation*, vol. 27, no. 2, pp. 186–192, 2009.

[31] M. Kmieciak, M. Gowda, L. Graham et al., “Human T cells express CD25 and Foxp3 upon activation and exhibit effector/memory phenotypes without any regulatory/suppressor function,” *Journal of Translational Medicine*, vol. 7, article 89, 2009.

[32] J. Wang, A. Iman-Facsinay, E. I. H. van der Voort, T. W. J. Huizinga, and R. E. M. Toes, “Transient expression of FOXP3 in human activated nonregulatory CD4+ T cells,” *European Journal of Immunology*, vol. 37, no. 1, pp. 129–138, 2007.

[33] S. E. Allan, S. Q. Crome, N. K. Crellin et al., “Activation-induced FOXP3 in human T effector cells does not suppress proliferation or cytokine production,” *International Immunology*, vol. 19, no. 4, pp. 345–354, 2007.

[34] V. Rigo, M. V. Corrias, A. M. Orengo et al., “Recombinant IL-21 and anti-CD4 antibodies cooperate in syngeneic neuroblastoma immunotherapy and mediate long-lasting immunity,” *Cancer Immunology, Immunotherapy*, vol. 63, no. 5, pp. 501–511, 2014.

[35] M. Croce, M. V. Corrias, A. M. Orengo et al., “Transient depletion of CD4+ T cells augments IL-21-based immunotherapy of disseminated neuroblastoma in syngeneic mice,” *International Journal of Cancer*, vol. 127, no. 5, pp. 1141–1150, 2010.

[36] W. Jing, J. A. Gershon, and B. D. Johnson, “Depletion of CD4+ T cells enhances immunotherapy for neuroblastoma after syngeneic HSCT but compromises development of antitumor immune memory,” *Blood*, vol. 113, no. 18, pp. 4449–4457, 2009.

[37] W. Jing, X. Yan, W. H. D. Hallett, J. A. Gershon, and B. D. Johnson, “Depletion of CD25+ T cells from hematopoietic stem cell grafts increases posttransplantation vaccine-induced immunity to neuroblastoma,” *Blood*, vol. 117, no. 25, pp. 6952–6962, 2011.

[38] M. V. Corrias, M. Occhino, M. Croce et al., “Lack of HLA-class I antigens in human neuroblastoma cells: analysis of its relationship to TAP and tapasin expression,” *Tissue Antigens*, vol. 57, no. 2, pp. 110–117, 2001.

[39] R. Castriconi, A. Dondero, M. V. Corrias et al., “Natural killer cell-mediated killing of freshly isolated neuroblastoma cells: critical role of DNAX accessory molecule-1-poliovirus receptor interaction,” *Cancer Research*, vol. 64, no. 24, pp. 9180–9184, 2004.

[40] M. Ponzoni, F. Guarnaccia, M. V. Corrias, and P. Corniglia-Ferraris, “Uncoordinate induction and differential regulation of HLA class-I and class-II expression by γ-interferon in differentiating human neuroblastoma cells,” *International Journal of Cancer*, vol. 55, no. 5, pp. 817–823, 1993.

[41] P. Engelmann, K. Farkas, J. Kis et al., “Characterization of human invariant natural killer T cells expressing FoxP3,” *International Immunology*, vol. 23, no. 8, pp. 473–484, 2011.