Chapter 2

The Origin of Neuroblastoma

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Abstract

It is widely accepted that neuroblastoma origin from Neural Crest Cells (NCC). NCC is a group of embryonic cells located in proximity to neural tube. During the embryonic development they migrate to generate the ganglia of sympathetic nervous system and the adrenal medulla. More than 50% of neuroblastoma masses are detected in the abdomen but the phases of tumorigenesis during the embryonic life are still unknown. Neuroblastoma cells show numerous copy number aberrations (CNAs), both numerical and structural. Several non-random CNAs are detected in clinical stage 4 and associated with tumor aggressiveness. On the contrary, neuroblastoma cells of infants or young patients have several numerical CNAs that are associated with a favorable outcome. *MYCN* oncogene amplification was one of the first genetic abnormalities observed in neuroblastoma and was found correlated to tumor aggressiveness. About 1% of all neuroblastoma show a hereditable fashion. Nowadays, the ALK gene has been discovered as predisposition gene for neuroblastoma. Moreover, thank to the genome-wide association studies, *BARD1, LMO1* and *LIN28* genes have been found linked to neuroblastoma predisposition. The two-step and multistep models are not satisfied the genesis of this tumor making the study of neuroblastoma tumorigenesis mandatory. Recently, the role of chromosome instability (CIN) became prominent to explain the neuroblastoma development. Indeed, the chromothripsis was observed in neuroblastoma cells of clinical stage 4, supporting the high genomic instability of these cells. The role of CIN in neuroblastoma is still unclear, but several experimental data suggest that CIN has a pivotal part in the genesis of neuroblastoma.

Keywords: neuroblastoma, neural crest cells, tumorigenesis, chromosomal instability, two-hit model, multistep model, chromosome instability

1. Introduction

The OMICs (the OMICs is a neologism referred to the study of: genomics, proteomics, transcriptomics, etc.) study of neuroblastoma has produced a huge amount of data generated by

genomics profiling, gene expression and epigenetics analysis [1]. Nevertheless, these studies supply information about the picture of primary tumors or metastatic cells only at onset of the disease. More recently, Schramm et al. [2] were able to study the genome of neuroblastoma comparing the mutational profiling of tumor cells at onset of disease and at time of patient's relapse. This allowed the authors to investigate the tumor clonal evolution and demonstrate that new mutations were acquired during the tumor progression. Recurrent mutations in tumor of relapsed patients included: cadherin 5 (*CDH5*), dedicator of cytokinesis 8 (*DOCK8*), protein-tyrosine phosphatase nonreceptor type 4 (*PTPN14*), Harvey rat sarcoma viral oncogene homolog (*HRAS*) and Kirsten rat sarcoma viral oncogene homolog (*KRAS*), providing a lot of information concerning new mutations present in the tumor of patient at relapse. Since patient's relapse is a critical step in the cure of neuroblastoma for the reason that, usually, tumor is not responding to the therapy, it is reasonable that new mutations contribute to increase tumor aggressiveness and drug resistance. *MYCN* oncogene amplification is one of the most important gene abnormalities found in neuroblastoma, and it is largely used as tumor unfavorable marker. The *MYCN* status (normal versus amplified) is used to classify the relapse patient's risk. Consequently, in the last decade, in order to perform precision medicine, particular attention was focused to find mutations candidate to drug targeting [3].

However, although the OMICs approach allowed us to identify some new drugs useful for target therapy, the cure of neuroblastoma is partially ineffective with a 5-year overall survival of 35% [4].

Actually, the overall OMICs studies are lacking important information about the origin of neuroblastoma. Really, we do not know how and when the mutations that we observed are occurring in neuroblastoma cells. Some animal models have been produced to recapitulate the growth and development of neuroblastoma, and some mathematical models have been generated to mimic the tumorigenesis of the tumor, but the overall information about the genesis of neuroblastoma is still missing. Certainly, the deep knowledge about the neuroblastoma tumorigenesis will greatly contribute to the cure of this pediatric cancer.

2. From neural crest cell to neuroblastoma cell

There are evidences that neuroblastoma tumor cells originate from neural crest cell (NCC) [5, 6]. Most of neuroblastoma cells produce homovanillic acid and vanillylmandelic acid, two metabolites involved in catecholamine synthesis of sympathetic nervous system [7, 8].

The neuroblastoma is comprised in Neuroblastic Tumors a group of tumors with great heterogeneous morphology [9]. Neuroblastoma cells show small cell body with few cytoplasm and abundant nucleus, while some neuritis protrudes by the cell body. Neuroblastic Tumors are classified as: Neuroblastoma Schwannian stroma-poor (undifferentiated, poorly differentiated, differentiating), Ganglioneuroblastoma intermixed Schwannian stroma-rich and Ganglioneuroma. Undifferentiated Neuroblastoma Schwannian stroma-poor is one of the

most aggressive Neuroblastic Tumors, whereas Ganglioneuroblastoma intermixed Schwannian stroma-rich and Ganglioneuroma are less aggressive. Ganglioneuroblastoma and Ganglioneuroma display heterogeneous morphology with large cells resembling ganglionic-like cells or Schwann-like cells. The latter are also identified as stromal cells. In **Figure 1**, the cell morphology of some Neuroblastic Tumors is shown.

It is also interesting to report that neuroblastoma cells of human-established cell lines produce a lot of long neuritis, creating a dense neural network after all-trans retinoid acid (ATRA) treatment [10, 11]. Moreover, the treatment with ATRA blocks the cellular proliferation inducing neuroblastoma cellular maturation. More than 50% of the patients onset with an abdominal mass, while other patients have head, neck and paraspinal infiltrated lymph-nodes [7]. Trunk NCC moves from dorsal to ventral region to form sympathetic ganglia and adrenal medulla.

It is still unclear if these cells are also a committee to become malignant neuroblastoma cells or if the malignant transformation is initiated after the NCC reached their final destination.

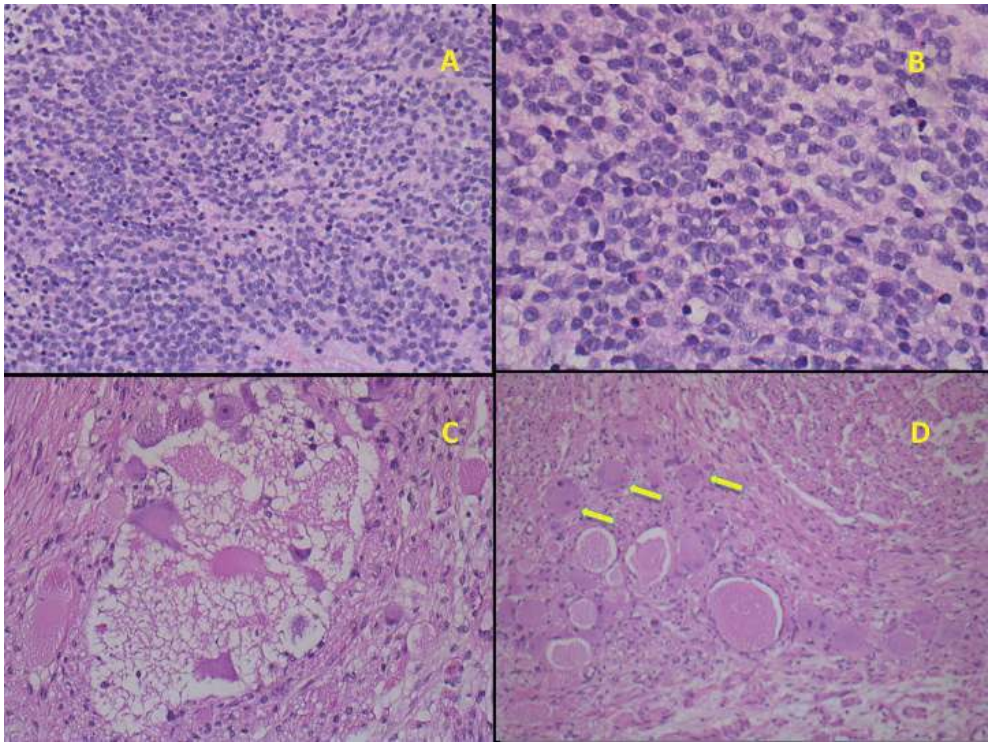

Figure 1. Morphological heterogeneity of neuroblastic tumors. (A) Poorly differentiated neuroblastoma stroma-poor (magnification 20x) and (B) same tumor (magnification 40x). Tumor tissue shows small blue cells with few cytoplasm and few stroma. (C) Ganglioneuroblastoma intermixed Schwannian stroma-rich (magnification 20x). Cells are embedded in abundant stroma. (D) Ganglioneuroma, very benign tumor; most of the tissue is stroma, and some ganglionic-like cells are visible (yellow arrow) (kindly provided by Dr. Luisa Santoro, University of Padua, Italy).

There are several evidences that neuroblastoma arises during fetal life. Ikeda et al. [12] have observed neuroblastoma cells in autoptic samples of infants. This observation clearly indicates that tumor may grow and develops during the fetal life. Patients with neuroblastoma stage 4S, a special group of patients that develop the tumor within one year of age and that may show onset of disease in the first month of life (**Figure 2**), are presumably developing the tumor already before birth. This has been demonstrated by Gigliotti et al. [13] who reported that six cases out of 45 stage 4S neuroblastomas were detected in utero. So that, we have several, indicating that neuroblastoma tumorigenesis may initiate during the fetal life.

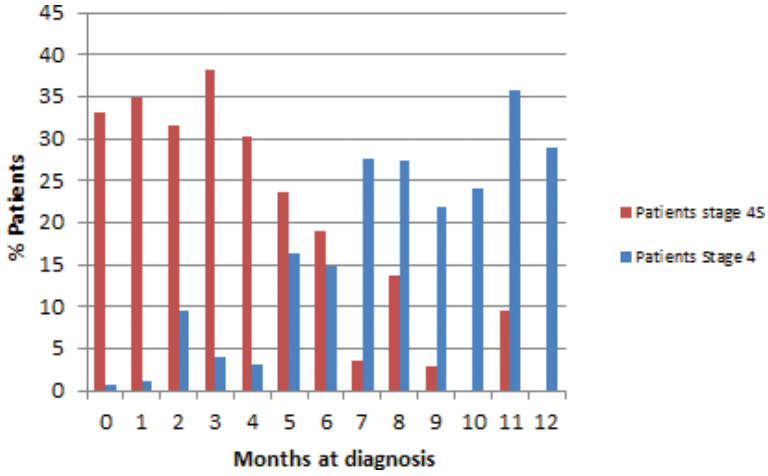

Figure 2. Distribution of stage 4S and stage 4 patients at onset during the first year of life. Most of stage 4S patients (red column) onset between 0 and 8 months, whereas patients at stage 4 (blue column) onset more frequently after 5 months [14].

3. Familial neuroblastoma and predisposition to neuroblastoma

About 1% of all neuroblastoma show familial cases. Genetic screenings of several families with recurrent neuroblastoma have shown that tumor is transmitted as recessive trait at low penetrance. In 2007, Longo et al. [14] have demonstrated a significant likelihood ratio for locus 2p by linkage analysis. Further, Mosse et al. [15] identified a locus at the chromosome region 2p23-24 with 104 genes that also included the *MYCN* gene. Next analysis showed significant mutations of Anaplastic Lymphoma Kinase (*ALK*) gene associated with the neuroblastoma predisposition. In neuroblastoma, *ALK* gene synthetizes the tyrosine alk receptor. Receptor autophosphorylation is promoted by mutation in kinase domain, and it is activating the alk pathway. Both germline and somatic *ALK* mutations were observed in neuroblastoma. Up to now, several mutations have been found in the kinase domain, but the most frequents are R1275 (43%), F1174 (30%) and F1245 (12%).

It is interesting to note that in human, both *MYCN* [16] and *ALK* [17] genes are highly expressed during the embryonic life and their expression decreases after the born. *MYCN* and *ALK* expression is not appreciable in the tissues of adult.

Recently, genome-wide association studies (GWAS) have been used to identify susceptibility gene variants in neuroblastoma. The GWAS studies are very useful to associate gene polymorphisms to tumor predisposition. Bosse et al. [18] identified the *BARD1 β*, an isoform of *BARD1* gene, associated with high-risk neuroblastoma. Oldridge et al. [19] found a polymorphism at locus of *LMO1* gene significantly associated with neuroblastoma susceptibility. In particular, they found the SNP rs 2168101, the variant most highly associated with the disease. A further association was found for *LIN28B* variants, making the *LIN28B* gene significantly involved in the neuroblastoma susceptibility [20].

4. Genomics and transcriptomics abnormalities in neuroblastoma cell

DNA content in neuroblastoma cells shows great variability. Most of the tumors with favorable evolution show triploid or near-triploid cells, whereas tumor of patients with unfavorable outcome shows diploid, near-diploid or tetraploid cells. Tumor of favorable cases has whole additional chromosomes and few structural damages, whereas tumor of patients with poor outcome usually has several nonrandom structural changes and few numerical chromosome aberrations [21–23].

Studies of neuroblastoma cell genome, by microarray comparative genomic hybridization, have shown the following nonrandom structural copy number aberrations (CNAs): deletions of chromosomes 1p36 region, 3p, 4p, 9p 11q 14q together gain of 1q, 2p24, 12p, 17q [21, 22, 24]. Structural CNAs are more frequently observed in tumor of patients with aggressive metastatic disease, and indeed, structural chromosome abnormalities are significantly associated with high tumor aggressiveness and disease progression [25]. This picture indicates that neuroblastoma cells have high chromosomal instability. Gain of chromosome 2p region is mainly due to the amplification of *MYCN* gene. The amplification of *MYCN* gene was discovered in 1984 and is the most robust genomic abnormality observed in about 20% of neuroblastoma [26]. Gene copies can range from four to over 1000, and *MYCN* gene amplification is significantly associated with rapid tumor growth and disease progression [27].

As well as CNAs, the gene expression of neuroblastoma cells was investigated by microarray technology. Gene expression profiling was found significantly different between localized and metastatic tumors. Oberthuer et al. [28, 29] have identified a 144-gene signature associated with tumor aggressiveness and poor patients' outcome. Moreover, a 59-gene signature has been also found associated with patient's risk although neither 144- nor 59-gene signatures are currently used as prognostic markers [30]. Recently, we have looked for the global gene expression between localized and metastatic tumors. This difference was calculated for each gene and called as transcription instability (TIN), and we generated a TIN-index that is significantly higher in metastatic tumor in respect of the localized one. Our study shows that gene expression is highly deregulated in advanced tumors [submitted manuscript].

5. One, two, multistep: the tumorigenesis of neuroblastoma

The tumorigenesis of neuroblastoma is still unclear. Neuroblastoma shows a great biological and genetics heterogeneity, suggesting that more than one gene is involved in the tumor initiation and progression. Neuroblastoma stroma-poor shows great potential of growth and rapidly forms metastases distally from primary tumor, indicating that gene mutations increase the tumor cell invasion capacity. On the contrary, ganglioneuroblastoma has low aggressiveness, indicating that not all gene mutations are critical for tumor growth and invasion. Ganglioneuroblastoma is composed by malignant neuroblasts and Schwannian stromal cells; the latter, possibly, are playing a tumor suppression activity by regulating the tumor microenvironment. Indeed, several observations report a complex cross-talk between neuroblastoma cells and Schwannian stroma cells, manly mediated by cytokines [31, 32]. Lastly, neuroblastoma cells of clinical stage 4S still are a fascinating problem, because this metastatic tumor can regress spontaneously. To explain disease regression of stage 4S tumor, it has been proposed that neuroblastoma cells are delayed to achieve the complete cell differentiation before the birth of patient, but the cell maturation is completed within the first year of patient's age [33].

Knudson and Meadows [34] have suggested the presence of a recessive NB gene to explain the behavior of stage 4S tumor, a model resembling the recessive RB gene discovered in retinoblastoma. Since chromosome 1p36 region has been found deleted in more than 40% of cases, this region was a candidate locus to contain NB-suppressor gene [35]. Indeed, if one NB gene would be located in the deleted chromosome 1p36 region, the two-hit model should be fit in the carcinogenesis of neuroblastoma. Unfortunately, linkage analysis of neuroblastoma pedigrees has not revealed NB-genes in the 1p36 region.

Recently, animal models that recapitulate the genesis of neuroblastoma are produced in mouse and in zebrafish. *MYCN* human oncogene has been overexpressed in transgenic mouse, demonstrating that tumor growth is under *MYCN* control. Similarly, neuroblastoma can grow in mouse overexpressing *ALK* or *LIN28*. In zebrafish model, the overexpression of *MYCN* gene blocks the development of sympathoadrenal precursor toward the chromaffin-like cells, inducing the formation of neuroblastoma cells in the interrenal gland. Neuroblastoma tumor growing in both mouse and zebrafish resembles human neuroblastoma in many of aspects such as cell morphology [36]. Zebrafish has been also employed to study the embryonic development of NCC. These cells can be easily identified by the expression of *SOX10* (Sry-box 10) and *Crestin* genes that are expressed in migratory NCC. So, the precise and detailed analysis of NCC development during physiological embryonic life of zebrafish can greatly help to generate new models for the study of neuroblastoma tumorigenesis.

Finally, since several chromosome regions are affected in neuroblastoma tumor, it is reasonable to think that numerous genes are impaired giving a sort of multifactorial or multistep damage to the normal cells [37]. Loss of chromosome 1p36 region, 3p, 4p, 9p 11q 14q together gain of 1q, 2p24, 12p, 17q, mutations of *ALK* and receptor tyrosine kinase (*AXL*) gene, and telomerase reverse transcriptase (TERT) re-arrangements may be seen as mutational steps, leading to the transformation of normal neural crest cell to neuroblastoma cell.

In this contest, the role of gene mutation seems not sufficient to transform the normal cell into neuroblastoma, at least for all Neuroblastic Tumors because the frequency of gene mutation in neuroblastoma, as well as other pediatric cancer, is dramatically lower than mutations observed in adult cancer. On the other hand, few genes can have critical damaging mutations that are dangerous for the pathways in which genes work, suggesting an oligogenic mechanism of neuroblastoma tumorigenesis [38].

6. Chromosome instability and neuroblastoma

The low number of mutations in neuroblastoma suggests that abnormalities in gene sequencing play a role in some but not in all neuroblastoma. The more aggressive tumors are belonging to patients over one year of age with metastatic disease; these patients with stage 4 disease have a median age of onset between four and five years. Differently from infants' patients, tumor cells of stage 4 patients over one year of age have several nonrandom structural CNAs. Others and we have suggested that tumor cells of these patients accumulate chromosome damages in a time-dependent manner [39, 40]. As consequence, this suggests a multistep manner of neuroblastoma development and it is reasonable to think that several alterations such as single gene mutation, gene deletion, gene amplification, gene rearrangement, and gross chromosome aberrations participate in the neuroblastoma tumorigenesis. This hypothesis is also supported by the discovery of chromothripsis, a catastrophic defect mainly occurring in chromosome 5 that damages almost all chromosome regions [41]. More recently, I suggested that chromosomal instability (CIN) plays a crucial role in the neuroblastoma development [42]. CIN is a feature of most cancers and can be caused by abnormal mitosis, failure in the microtubule and centrosome dynamics, or spindle apparatus, abnormal control of double-strand break repair, telomere maintenance, and abnormal telomere function. Several genes are involved in the mentioned mechanisms, and so they can be considered CIN-related genes. Carter et al. [43] have proposed two CIN: CIN25 and CIN70 (the number shows the number of genes considered in the signature) expression signatures that were used to predict survival in lung adenocarcinoma, medulloblastoma, breast cancer, lymphoma and other tumors. Unfortunately, neither CIN25 nor CIN70 was a good prognostic marker in neuroblastoma. **Table 1** shows some genes included in the CIN signatures together with genes that are involved in the mitotic control and that can contribute to CIN after their deregulation. The complexity of CIN is shown by the presence of at least two CIN: the whole chromosome instability (W-CIN) characterized by additional entire chromosome and the segmental chromosome instability (S-CIN) that shows chromosome structural changes such as deletion, amplification and rearrangements.

In neuroblastoma, we often found W-CIN in localized stage 1 and 2 tumors, while both W-CIN and S-CIN are detectable in tumors of stage 3, 4 and 4S. How W-CIN and S-CIN can contribute to the tumorigenesis is still unclear. A mathematical model [44] indicates that abnormal numerical whole chromosome number occurs very early in stage 4S (**Figure 3**). Additional whole chromosome aberration is followed by segmental chromosome damage in stage 4 tumors. So, we can argue that W-CIN is beginning in stage 4S and W-CIN together with S-CIN is present in stage 4.

Gene	Locus	OMIM	Function
ATRX	Xq21.1	300032	Associated with pericentromeric heterochromatin during interphase and mitosis; ATRX protein is also associated with chromosomes during mitosis
AURKA	20q13.2	603072	Induction of centrosome duplication-distribution abnormalities and aneuploidy
AURKB	17p13.1	604970	Direct interaction between CENPA and AURKA
BRRN1	2q11.2	602332	Involved in interphase and mitotic condensation
BUB1	2q13	602452	Protects centromeric cohesion during mitosis throughout SGO1
CNAP1	20q11.22	609689	Human rootletin (CROCC) bound CNAP1 at the proximal end of centrioles and formed the bridge between two centrioles
CDCA8	1p34.3	609977	CDCA8 interacts with AURKB and survivin (BIRC5)
CDC2	10q21.2	116940	Coordinates spindle assembly with the cell cycle during mitosis through phosphorylation of RCC
CDC20	1q34.2	603618	CDC20 together with HCDH1 active APC during mitosis and G1
CCNB1	5q13.2	123836	CCNB1 with p34 (cdc2) (CDK1) form a mitosis-promoting factor
CENPE	4q24	117143	Associates with kinetochores
ECT2	3q26.31	600586	ECT2 is localized in central spindle at anaphase and promotes local activation of RhoA GTPase
ESPL1	12q13.13	604143	Loss of sister chromatid cohesion activity
FOXM1	12p13.33	602341	Regulates expression of cell cycle proteins
H2AFX	11q23.3	601772	Involved in chromatin conformation
MAD2L1	4q27	601467	Induction of chromosome instability by overexpression of the mitotic checkpoint gene Mad2
NEK2	1q32.3	604043	Overexpression of active NEK2 induces a splitting of centrosomes
KIF4A	Xq13.1	300521	In HeLa cells, KIF4 interacted directly during spindle midzone formation
PTTG1	5q33.3	604147	PTTG1 appeared to be an anaphase-promoting complex
PRC1	15q26.1	603484	In HeLa cells, PRC1 together KIF4 interacted directly during spindle midzone formation
TOP2A	17q21.2	126430	Involved in chromosome segregation
TPX2	20q11.21	605917	Involved in microtubule organization during mitosis and normal spindle morphology
TTK	6q14.1	604092	Regulator of genetic stability and spindle apparatus

In the Table are listed genes participating in the CIN. The chromosome locus, OMIM code and function are reported for each gene. Some genes such as *AURKAB, BUB1, CNAP1* and *CDCA8* cooperate with other genes in the control of genetics stability and mitotic checkpoint (derived from Refs. [43, 45]).

Table 1. Genes involved in CIN.

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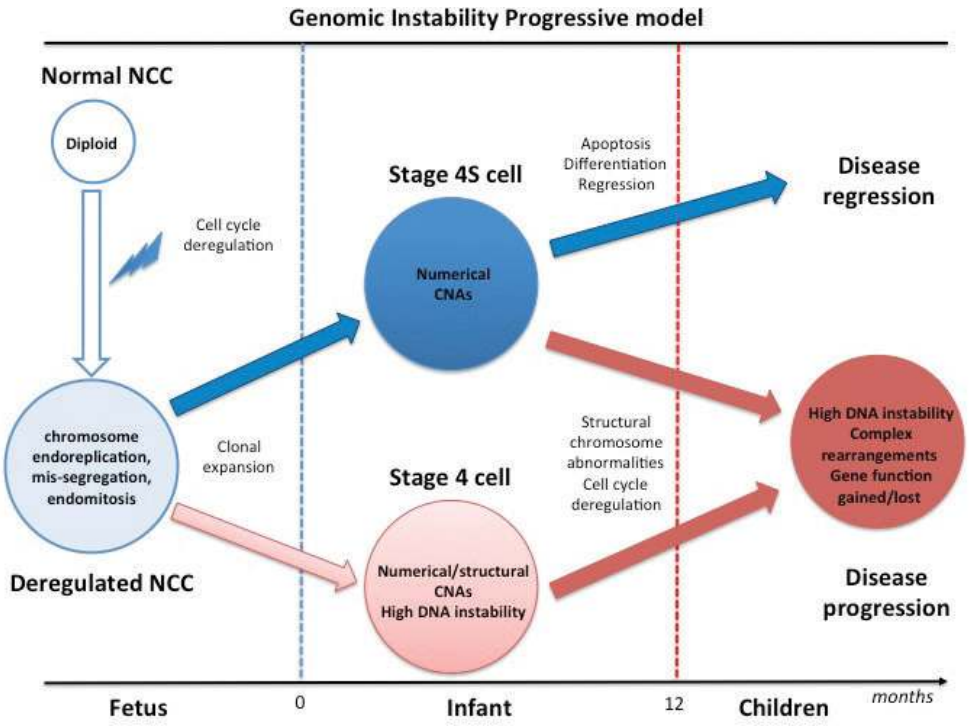

Figure 3. Model of genomic instability in neuroblastoma. The model suggests that tumor of stage 4S and 4 originates from NCC by numerical copy number abnormalities (CNAs) for stage 4S and numerical together with structural CNAs for stage 4. After infant life, only tumors with high genomic instability either stage 4S or stage 4 develop, contributing to disease progression [44].

As underlined before, Neuroblastic Tumors show biological and morphological heterogeneous features. It is possible that genotype-phenotype aspects reflect diverse road of the genesis of this tumor in which different genes are involved with different critical damages. This scenario makes the study of neuroblastoma tumorigenesis very complex.

7. Conclusion

Today, we have a huge amount of biological and clinical data of neuroblastoma. We know genome mutations and gene expression abnormality occurring in neuroblastoma cells. However, all data are referring to the tumor cells at onset of the disease and in same cases at relapse. Up to now, we have no information about the tumorigenesis of the tumor. The initial phases of tumor growth and progression are very important for the diagnosis and treatment of neuroblastoma. Very few mutations are present in this pediatric cancer, and the mutational two-step or multistep models do not completely fitting with the neuroblastoma

tumorigenesis. We develop a mathematical models to explain the genesis of this tumor. Moreover, we have several information about the chromosome damage and chromothripsis, suggesting that CIN plays a crucial role in neuroblastoma tumorigenesis. Some animal models seem to recapitulate the development of the tumor, focusing our attention to few crucial genes. However, we are still in the dark zone and the origin and development of neuroblastoma remains unsolved.

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