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Literature, Russian Literature

AbstractNeuroblastoma (NB), a developmental cancer, has poor outcomes, emphasizing the need to understand its pathogenesis. The heterogeneous aswell as clinical phenotype of NB is due to the biological and genetic featuresranging from rapid progression to spontaneous regression. Currentclassification of NB Tumours is based on MYCN oncogene amplification andattempts have been made to improve classification of NB based onmethylation of single-genes, or genome-wide profiles.

Despite recentgenome-wide mutation analyses, most of the primary NBs do not harbordriver mutations, implicating epigenetic-mediated gene regulatorymechanisms in the initiation and maintenance of genetically defined NB. Aberrant epigenomic mechanism, as demonstrated by global changes in DNAmethylation signatures, acetylation, re-distribution of histone marks andchange in the chromatin architecture might lead to NB. Here we review thecurrent understanding of epigenetic alterations, particularly at the DNA andhistone level, and their potential application for diagnosis, prognosis andtreatment of NB. 1.

DNA methylation in NeuroblastomaDNA methylation, being one of the essential epigenetic mechanisms, isclosely correlated with the process underlying cell growth, differentiation andtransformation 1. DNA methylation attenuates gene expression by the additionof methyl groups to cytosine residues within the CpG-rich sequences, presentin the promoter region of genes. Even though detailed regulatory mechanismsfrom DNA methylations remains to be elucidated the regulatory effect onexpression patterns in many of the cancers have been identified. For instance, aberrant DNA

methylations at promoter CpG islands are generally associatedwith reduced transcriptional activity and silencing of Tumour suppressorgenes thereby contributing to induction of malignancy2, 3, 4.

However, DNAmethylation at non- CpG sites has also been reported in cancer cells andstudies by Gómez et al in a small cohort of patients with NB identified thepresence of non-CpG methylation sites and their association withdifferentiation and expression of some of the key genes involved in NB suchas ALK 5. Incidentally, during oncogenesis, methylations at non-CpG sites areasymmetrical and are not widely distributed, hence methylated CpG sites stillrepresent the main epigenetic determinants in cancer. Genome-wide DNAmethylation levels are now increasingly required to determine the epigeneticevents involved in NB tumourigenesis. 1a. DNA methylation profiling of primary Neuroblastoma tumours andcell linesTo identify epigenetic deregulation mechanism in NB tumourigenesis, Charletet al compared the methylation pattern of NB cell lines to human neural crestprecursor cells, using promoter and CpG island microarrays.

In their analysis, among hypermethylated genes, MEGF10, a cell engulfment and adhesionfactor gene, was epigenetically repressed in the NB cell lines. MEGF10expression was also found to be significantly down regulated in NB Tumoursamples and had reduced relapse-free survival. Also, knock down of MEGF10in NB cell lines in vitro promoted cell growth and proliferation indicating anepigenetic mark that maintained the silenced state of this gene6 although invivo studies are needed to validate the effect of MEGF10 in NB. Methylationpatterns can also be used to distinguish NB tumour into

clinically relevantgroup. In study by Olsson et al, methylations of highest number of genes atCpG site had been identified in metastatic NB. TERT, telomerase reversetranscriptase, had the highest number of hypermethylated sites and wasfound to be associated with tumour progression and poor prognosis in NB andhas been proposed as a biomarker for risk stratification of NBs 3.

Rauschertand colleagues reported an epigenetic mechanism underlying the silencing ofLamin A/C, which provide nuclear assembly, chromatin organization, andtelomere dynamics, by CpG promoter hypermethylation in a subset of NBcells leading to enhanced tumour properties of NB. The effect ofhypermethylation was evident when Lamin A/C was reintroduced in the celllines deficient in this gene which induced a slow cell growth kinetics, delayedmigration, invasion, and colony formation together with cytoskeletalreorganization in NB 7. Targeting Lamin A/C in NB might have an impact sincetumour cells deficient in Lamin A/C exhibits more aggressive behaviour.

In arecent study by Henrich et al, PCDHB (Protocadherin Beta Cluster)methylation patterns were identified in patients who were associated withhigh-risk NB. Hyper-methylation of PCDHB resulted in down-regulation oftheir transcripts which contributed to NB development 8Among different stages of NB, special stage IV NB (Stage 4S) presents anintriguing condition where infants diagnosed with metastases are associatedwith an excellent outcome, due to its ability to undergo spontaneousregression9. In order to understand the phenomenon of spontaneousregression, Decock et al profiled

the promoter methylome of stage 4S NBpatients using methyl-CpG-binding domain (MBD) sequencing analysis inprimary tumour samples.

DNA methylation pattern of stage 4S NB, whencompared with stage 1/2 and stage 4, is dominated by differential methylationof target genes of several transcription factors that are involved in neural crestdevelopment and neural differentiation like MSX1, EVI1, E2F1, EGR3, AHR, MEF2A, YY1, PPARA, POU2F1, GFI1 etc. SLC9A5, a target gene of E2F1and MEF2A was found to be hypermethylated in stage 4S while low SLC9A5expression levels were seen in stage 1/2 and stage 4 tumours. These findingsshowed a characteristic methylation pattern of stage 4S NB compared tostage 1/2 and stage 4 tumours and they arise during different phases ofneural crest cell development 10. Another feature of high-risk NB is the high level of DNA methylation ofputative tumour suppressor genes. Extensive perturbations of DNAmethylation cause changes in gene regulation that promote oncogenesis.

Studies on number of candidate genes have revealed that hypermethylationdrives the NB oncogenic process4. Epigenetic silencing of tumour suppressorgenes of RASSF gene family members, RASSF2, RASSF4, RASSF5, RASSF6, RASSF7, and RASSF10, have been found to be frequent in NB celllines and primary tumours and considered to be involved in poor prognosis. Treatment with 5-Aza-dC (DAC), an epigenetic modifier that inhibits DNAmethyltransferase activity, in NB cell lines restored the RASSF geneexpression by blocking RAS induced apoptosis in NB cancer cells 11. Severaltumour suppressor genes have been reported to be epigenetically

silenced byDNA methylation in NB tumour and cell lines (Table 1). Table 1: Summary of TSGs silenced by promoter methylation in NBWhole-genome methylation profiles of NB tumours, showing a high degree ofclinical heterogeneity, matched with normal tissue from NB patients have ledto the identification of a large amount of putative prognostic biomarkers, butonly very few have shown clinical validity and utility due to inadequate studydesign, insufficient cohorts not enough to be of statistical significance and lackof biomarker validation thus falling short in covering NB heterogeneitylandscape23. Decock et al performed methyl-CpG-binding domain sequencinganalysis in 87 primary tumours, which is adequate given that most of thereported studies have relatively limited number of tumour samples, and twoindependent cohorts of 132 and 177 primary tumours were used to identifyNB specific prognostic biomarkers. They identified novel prognosticmethylation biomarkers; CCDC177, NXPH1, SPRED3, TNFAIP2, NPM2 forNB event-free survival and CYYR1 for overall survival.

Interestingly, most ofthe genes identified in the analysis are linked to neuronal process24. Table 2list the prognostic biomarkers of NB identified by methylation profiling . Table 2: List of prognostic methylated biomarkers identified in NB1b. miRNAome methylation in NeuroblastomamiRNAs are highly conserved and involved in different biological processeslike cell proliferation, apoptosis, migration and differentiation.

In a pathologicenvironment, miRNA dysregulation contributes to phenotypic alterationswhere it can act either as tumour suppressor genes or oncogenes accordingto the function of the proteins encoded by the target gene28. One

of thecharacteristic features of miRNA is that they can influence gene expressionwithout altering the DNA sequence making it an integral component of theepigenetic machinery. DNA methylation of miRNA indirectly influences the upor down regulation of target genes which results in the hypo- or hypermethylationof miRNAs respectively29.

Coordinated actions of miRNAs andother epigenetic factors regulate several biological processes where miRNAscan repress the expression of epigenetic factors or cooperate to modulatecommon targets. Most of the miRNA genes have CpG sites and are regulatedby DNA methylation in tumours and in a cancer-specific condition, such asmiR-31 in breast cancer 30. Parodi et al studied the complex network between miRNAs and genesinvolved in cell cycle and apoptosis pathways in NB. DNA methylationscreening in regulatory regions of miRNAs involved in those pathwaysrevealed potential methylation targets in NB namely cluster 34b/c, cluster23b/24-1/27b, miR- 124, miR -149, miR- 155 and miR- 196a1 in NB cell lines. DNA methylation analysis in tumour samples of NB patients also confirmedthe presence of hypermethylation for cluster 34b/c and miR- 124 which mightplay a role in NB aggressiveness.

This study revealed the presence ofepigenetic dysregulation which contributed to the functionality of cell cycle andactivation of apoptosis pathway in NB 31. Maugeri et al investigated the role ofpromoter methylation in miRNAs encoding genes in NB. They profiled 754miRNAs of specific CpG islands using methylation assays and in silicoanalyses. Promoter

encoding miR-29a- 3p, which is known to be downregulated in NB, have methylated CpG islands which decreased on treatmentwith 5′-AZA32.

Functional studies have determined that several of thehypermethylated miRNAs, as listed on table 3, target a large repertoire ofgenes that are over-expressed in NB tumours with substantial redundancywhich negatively impact NB cell proliferation and migration, both in vitro and invivo. Das et al investigated the coordinated miRNA and DNA methylationchanges in regulating NB cell differentiation by using all trans-retinoic acid(ATRA), which cause NB cell lines to increase in neurite length during theprocess of neural cell differentiation. They identified demethylation of methyltransferases, DNTMT1 and DNTMT3, along with upregulation of miRNAstargeting them, such as miR-152 and miR-26a/b following the ATRAtreatment33. Table 3 list the hypermethylated miRs and their target genesidentified in NB.

Table 3. Hypermethylated miRs and their targets in Neuroblastoma2.

Epigenetic therapy in NBAs normal cells undergo malignant transformation, epigenetic modifiers suchas DNA methyl transferases (DNMT) and histone deacetylases (HDACs)maintain the modification status of gene loci in tumour cells39. Usingdemethylating agents and histone deacetylase inhibitors demonstrate thatgenes such as tumour suppressor genes can be re-expressed in cell lines buttheir impact on clinical trials are still on-going. Despite the challenging aspects, advances have been made in identifying the potential role of epigenetictherapies in NB.

At present, it is not yet considered standard of care andcombination of agents with chemotherapy might improve sensitivity to NBtreatment. 2. 1 Drugs targeting DNA methylations in NBUnlike genetic alterations, DNA methylation can be reversed to restore thefunction of key control pathways in malignant and premalignant cells bytreatment with demethylating agents such as DNA methyltransferaseinhibitors (DNMTi) namely azacitidine (5-azacitidine) and decitabine (5-Azadeoxycytidine)which induces functional reversion of aberrantly silencedgenes in cancer39. These classes of inhibitors are now being evaluated inPhase 1 clinical trials in combination with other agents in patients with NB. 5-Aza-deoxycytidine (AZA) was used in clinical trial, as an anticancer drug forpatients with NB, but clinically relevant biologic effects was not well toleratedwith AZA causing significant myelosuppression40. One of the limitations ofAZA in NB is it’s a poor activator of tumour suppressor genes. Thoselimitations were profounded by studies by Westerlund et al where theycombined AZA and differentiation-promoting retinoic acid (RA) which impededNB growth and induced the expression of HIF2?, a tumour suppressor gene. This combination approach targeted high-risk NB responding poorly to RAtherapy41.

Another group reported treatment of AZA and tamibarotene (TBT), a synthetic retinoid, in a panel of NB cell lines, which supressed proliferationand induced an increase in the number of cells in S phase. Combination ofAza- and TBT was investigated in vivo in a mouse xenograft model, whichresulted in significant tumour regression without severe side-effects42. 2. 2 Histone modifications (methylation / demethylation/

acetylation/deacetylation) as drug target for NBWell-known histone modifications that are found to be involved in regulatinggene expressions are methylation, demethylation, acetylation anddeacetylation. Histone methylation (HM) is involved in gene transcription andchromatin remodelling and is linked to inactivation of a number of criticaltumour suppressor genes. Histone methylation is considered as an epigeneticmark that is dynamically regulated by histone methyltransferases anddemethylases.

Histone methyl transferases (HMT), which catalyses histonemethylations, are one of the widely studied chromatin modifying enzymes andconsidered as a potential therapeutic target. Numerous studies have beenreported for HMT inhibitors in NB cell lines to determine its effect in cellproliferation and migration. In one of recent studies, treatment with the smallmoleculeinhibitor, SGC0946, which targets DOT1L, a histonemethyltransferase that catalyzes methylation at the H3K79 position, in NBcells reduced H3K79 methylation and down regulation of MYCN, ODC1andE2F2 genes which reduced NB cell proliferation43. However, in vivo studiesare needed to determine the efficacy of these inhibitors of HM in NB. Recently, Veschi et al identified SETD8, methyl transferase which catalyse methylationof H4K20, as a crucial regulator of cell growth and differentiation in high-riskNB. Pharmacological inhibition of SETD8 by UNC0379 in NB cell linesinduced SETD8 knockdown and effectively inhibited the proliferation of cells invitro and in ex vivo models44. Xue ke et al studied role of G9a, methyltransferase for H3K9, along with G9a inhibitor BIX01294 in xenograft mouseNB mouse model.

The treatment with the inhibitor BIX01294 resulted in thereduced tumour volume in NOD/SCID mice which rendered the possibility asa potential therapeutic target45. Histone lysine demethylases (HDM) induces the expression of oncogenictranscription factors including MYC46. Histone demethylase family with diversefunctions are implicated in regulation of NB cell survival. Therefore, therapeutic activity could be achieved by targeting histone demethylaseswhich might block the expression of oncogenic transcription factors like MYCand activate tumour suppressive pathways in NB. Recently, Yang et alidentified novel histone demethylase inhibitor, ciclopirox that binds KDM4B, one of the families of HDM, and inhibited NB growth and metastasis in adisseminated disease model of NB47. The study indicates that pan-KDMinhibition in NB clinical trials might contribute to its overall anti-tumour effect.

Lysine-specific demethylase 1(LSD1), which catalyses lysine demethylation, physically bind to MYCN both in vitro and in vivo. Combined pharmacologicalinhibition of MYCN and LSD1 by TCP and 10058-F4 respectively reducedMYCN-amplified NB cell viability in vitro. The ability of these inhibitors tospecifically inhibit the function of both genes is of great importance and couldlead to development of novel therapeutic approaches to treat MYCN-inducedNB48. Histone deacetylases (HDACs) enzymatically remove the acetyl group fromhistones and regulate gene expression.

Histone deacetylase inhibitors(HDAC) are class of epigenetic modifiers that activate silent genes such ascyclin-dependent kinase by altering the acetylation state of histone tails39. HDAC inhibitors block the activity of

HDAC isozymes involved in numerousbiological processes although potential for toxicities that result in dose-limitingside effects were reported for pan-HDAC inhibition49. Rettig and colleaguesreported selective inhibition of one of the HDAC family, HDAC8, which arehighly expressed in metastasized NB tumours, which were rendered effectiveand less toxic than the unspecific inhibition of several HDAC family membersin a preclinical model of NB50.

HDAC-selective targeting might be an effectivetherapeutic in tumours exhibiting HDAC isozymes and could be combinedwith differentiation-inducing agents like RA. One group investigated thepotential activity of a HDAC inhibitor, MS-275, in combination with a pancarbonicanhydrase inhibitor, acetazolamide (AZ) in a pre-clinical NBxenograft model. On co-treatment, cancer stem cell genes (OCT4, SOX2 andNANOG) were found to be downregulated, which indicated the elimination ofthe NB-CSC properties. The combination treatment drastically reducedtumour growth in vivo and presents a future therapeutic potential of HDACinhibitors in patients with NB51.

Combination studies of RA with histonedeacetylase inhibitor trichostatin A, (TSA), resulted in anti-tumorigenic effectin SH-SY5Y and SK-N-BE cells and that combined therapy could be useful toinhibit NB progression 52Histone acetylation is important in differentiation and proliferation, signaltransduction, metabolism and cytoskeleton dynamics and initiated by theactivity of histone acetyltransferases (HATs), involved in acetylatingconserved lysine residues by transferring an acetyl group from acetyl-CoA toN-acetyl-lysine. Histone lysine acetylation is involved in

epigeneticmodifications that impact on gene expression and transcriptional activity53. Pyridoisothiazolone HAT inhibitors, PU139 and PU141 have been found toinduce cellular histone hypoacetylation and inhibit growth of NB cell lines. Both of the agents were able to block growth of SK-N-SH NB xenografts inmice due to the reduction of histone lysine acetylation54. The effect of theseagents needs to be scrutinised more in clinical trials in NB patients. ConclusionIn conclusion, the integration of genomic and epigenetic data provides strongevidence that DNA methylation and chromatin-based mechanisms are highlyderegulated in NB. The epigenetic processes leading to NB interact with eachother rather than operating independently thereby establishing a multilevelregulatory network altering the expression of tumour suppressive genes.

Ourincreased understanding of the epigenetic alterations that drive NB suggestnovel avenues for treatment, but extensive basic and clinical studies areneeded to translate these findings into favourable patient outcome.