Accepted Manuscript

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PII: S0303-7207(17)30343-X

DOI: 10.1016/j.mce.2017.06.016

Reference: MCE 9988

To appear in: *Molecular and Cellular Endocrinology*

Received Date: 28 February 2017

Revised Date: 28 May 2017

Accepted Date: 15 June 2017

Please cite this article as: Björklund, P., Backman, S., Epigenetics of pheochromocytoma and paraganglioma, *Molecular and Cellular Endocrinology* (2017), doi: 10.1016/j.mce.2017.06.016.

Epigenetics of Pheochromocytoma and paraganglioma

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Abstract

Pheochromocytomas and paragangliomas (PPGLs) are neuroendocrine tumors arising in the medullae of the adrenal glands or in paraganglia. The knowledge of the tumor biology of these lesions has increased dramatically during the past two decades and more than a dozen recurrently mutated genes have been identified. Different clusters have been described that share epigenetic signatures. Mutations in the succinate dehydrogenase complex subunit genes play a pivotal role in reprogramming the epigenetic state of these tumors by inhibiting epigenetic regulators such as TET enzymes and histone demethylases. Another subgroup of tumors carries hypomethylated genomes, and overexpression of several micro-RNAs has been described. While much remains to be investigated regarding the epigenetics of PPGLs, it is clear that it plays an important role in PPGL biology.

Keywords: Pheochromocytoma, Paraganglioma, Epigenetics, Adrenal, Methylation

Highlights

- Pheochromocytomas and paragangliomas carry epigenetic aberrations
- Succinate dehydrogenase mutations cause epigenetic dysregulation
- Several genes involved in epigenetic modulation have been described mutated in pheochromocytomas and paragangliomas

1. Introduction

The adrenal medullae are neuroendocrine tissues that secrete catecholamines during activation of the sympathetic nervous system. They are related to the sympathetic paraganglia, which are neuroendocrine tissues located in close proximity to sympathetic ganglia. The parasympathetic paraganglia, in contrast, are non-chromaffin and include the chemoreceptor organ in the carotid body. Pheochromocytomas and paragangliomas (PPGLs) are rare neuroendocrine tumors (incidence 2-8/1000000/year) that arise in these three tissues[1,2] with pheochromocytomas being those that occur in the adrenal glands and paragangliomas being those that develop at other sites. Most PPGLs are benign and can be cured by surgical resection. Ten to twenty percent of the afflicted patients develop metastases in organs normally devoid of chromaffin cells and are considered to have malignant PPGL. Due to the rarity of malignant PPGL, there is little evidence supporting any specific treatment regimen. Nevertheless, CVD (cyclophosphamide, vincristine and dacarbazine)[3,4], temozolomide[5] and sunitinib[6,7] have all been reported to have some anti-tumoral effects in small case series, and a phase II trial supports the use of MIBG in selected patients with metastatic disease[8].

Up to 40% of pheochromocytomas and paragangliomas are thought to be caused by germline mutations in at least 12 different well-characterized genes[2]. Mutations in *RET* cause Multiple Endocrine Neoplasia type 2[9,10] of which pheochromocytomas are a component. Von Hippel-Lindau's disease caused by inactivating mutations in *VHL* may also involve pheochromocytomas or paragangliomas[11], as may rare cases of Neurofibromatosis type 1[12] caused by mutations in the tumor suppressor *NF1*[13]. Additionally there are specific

paraganglioma syndromes caused by mutations in genes encoding Krebs' cycle enzymes (succinate dehydrogenase subunits: *SDHA [14]*, *SDHB* [15], *SDHC* [16], *SDHD* [17], *SDHAF2* [18] and fumarase: *FH* [19]). These tumors may also be caused by mutations in *TMEM127* (encoding an endosomal protein with an apparent role in regulating mTOR signaling) [20,21], *MAX* (Myc-Associated Factor X)[22] and by mosaic mutations in *EPAS1* (which encodes the Hypoxia Inducible Factor 2α) [23]. Recurrent somatic mutations have been reported in several genes including proto-oncogene *HRAS* [24], *NF1* [25,26], chromatin-remodeler *ATRX* [27] and *CSDE1* (which encodes an RNA binding protein)[28]. Fusion genes involving *MAML3* (encoding Mastermind Like protein 3) have recently been reported to occur in 5% of cases, and to occur in tumors that lack mutations in other known driver mutations [28].

In 1942 Conrad Waddington coined the term epigenetics, describing the phenotype resulted by interaction between environment and genotype[29]. Effect of environmental changes on inherited phenotype alterations in offspring, including (but not restricted to) maternal nutrition during pregnancy[30], general famine[31], early prenatal stress[32] and smoking[33,34] have been described. Albeit several definitions prevail and a common consensus is yet not achieved, epigenetics is commonly referred to inheritable functional changes not encoded by the linear DNA sequence, such as chemical modification of the DNA molecule, including methylation of cytosine residues [35], methylation of adenosine residues [36] as well as covalent modifications of the proteins that associate with DNA in chromatin: histones[37].

The established dogma of these modifications are that increased methylation of gene promoters lead to decreased expression of the associated gene[35], as does trimethylation of the Lys27 residue of histone H4 (H3K27me3)[38] while

decreased metylation of gene promoters[39], trimethylation of the Lys4 residue (H3K4me3) and acetylation of the histone[40], are associated with transcription. DNA methylation in non-promoter segments of the "high level" transcribed genes are generally associated regulation of splicing variants[41] and is associated with H3K36 methylation to inhibit activation of cryptic start sites[42].

In addition alterations caused by long non-coding RNA (lncRNA)[43] and and microRNAs (miRNAs)[44] as well as methylation of RNA[45] are considered as epigenetic traits.

2. DNA methylation in pheochromocytomas and paragangliomas

Several studies have investigated the existence and role of DNA methylation aberrations in PPGL. Early studies investigated the promoter methylation levels of selected candidate genes[46,47,48]. One such study provided the first evidence of more profound and wide-spread epigenomic dysregulation in PPGL; a CpG-island methylator phenotype (CIMP) was discovered and found associated with earlier onset of disease and aggressive disease with distant metastases[46]. Of note, all *SDHB*-mutated tumors in the cohort exhibited this phenotype. Several years later this result was corroborated when Letouzé et al. used an array-based approach to identify the same CIMP phenotype in *SDHx*-mutated tumors[49]. Letouzé et al. identified two additional methylation phenotypes in PPGL: a hypomethylated phenotype and a group of tumors with intermediate methylation levels. The hypomethylated tumors predominantly harbored mutations in genes involved in protein kinase signaling, whereas the intermediate group was enriched in *VHL*-mutated tumors. The hypomethylated and the intermediate groups have been validated in an independent study[50]. Hypomethylation has been experimentally linked to chromosomal instability[51], and the hypomethylated cluster

of PPGLs has been reported to carry a greater number of somatic copy number alterations[50].

DNA methylation is regulated by a number of enzymes including the DNA methyltransferases that establish DNA methylation, and the TET (ten-eleven translocation) family of enzymes which oxidize 5-methylcytosine to 5-hydroxymethylcytosine and further to 5-formylcytosine and 5-carboxycytosine, which can be removed and replaced by base excision repair in an active demethylation pathway [52]. Succinate and fumarate, which accumulate in succinate dehydrogenase deficient cells and fumarase deficient cells, respectively, have been demonstrated to inhibit the TET enzymes by binding to the iron-sulfur binding site[53]. This prevents the iterative oxidation of 5- methylcytosine, and thus inhibits the active demethylation pathway and leads to global hypermethylation. The same mechanism has been demonstrated to establish the CIMP-phenotype in *IDH1*-mutated glioblastoma tumors, in which 2-hydroxyglutarate accumulates and inhibits TET enzymes[54]. In *SDHB* deficient cells succinate accumulation and hypoxia inhibit α-KG-dependent dioxygenases, resulting in hypermethylation of DNA and histones [55].

Hypermethylation of certain genes in *SDHB*-mutated PPGLs has been proposed to play a role in their propensity for malignant behavior by activating epithelial-to-mesenchymal transition (EMT) pathways[49,56]. Tumors with these mutations are known to exhibit gene expression patterns associated with EMT including overexpression of TWIST and matrix-metalloproteases (MMPs), and underexpression of CDH2 (Neural Cadherin, which plays a role in cell adhesion) and KRT19 (cytokeratin 19, a component of the cytoskeleton) [56]. *KRT19/Krt19* has been shown to be hypermethylated and down-regulated both in *SDHB*-mutated human PPGLs and in an *Sdhb*-null immortalized murine chromaffin cell line [56]. The *Sdhb*-null cell line also

displays an EMT-like phenotype with a migrating behavior, which is decreased upon infection with a lentivirus carrying Krt19 cDNA[56]. This suggests that aberrant DNA methylation may play an important role in establishing the aggressive phenotype often observed in *SDHB*-mutated tumors.

The methylation level of specific CpG residues has been suggested as a marker of malignant disease[57]. High methylation-levels of the promoter for *NELFE* (which is also known as *RDBP* and encodes the Negative Elongation Factor Complex Member E) in particular was suggested to predict malignant behavior regardless of genotype. This was not confirmed in a smaller cohort without tumors with the CIMP phenotype[50]. Further studies are needed to clarify the potential role of DNA methylation analyses in a clinical context.

Furthermore, DNA methylation has been linked to therapeutic response in metastatic PPGL. O6-methylguanine-DNA methyltransferase (encoded by the *MGMT*) gene is involved in removing alkyl groups from modified guanine residues in DNA. Consequently it plays a role in repairing the damage to DNA caused by alkylating chemotherapeutic agents. Epigenetic inactivation by hypermethylation of the *MGMT* promoter is related to good response to temozolomide in malignant primary brain tumors and is used by clinicians to guide therapy[58]. A study investigating the efficacy of temozolomide in patients with metastatic PPGL found partial response only in patients with *SDHB*-mutations[5]. *SDHB*-mutations were associated with hypermethylation of the *MGMT*-promoter and low expression of the MGMT protein, which at least partially explains this finding.

3. Epigenetic driver mutations

In addition to the more peripheral and disease modifying role presumably played by alterations in DNA methylation, there is some evidence that epigenetic alterations and alterations in epigenetic regulators may drive the development of pheochromocytoma and paraganglioma. A recent case report described epigenetic inactivation of *SDHC* by promoter hypermethylation in a patient with multiple paragangliomas[59]. RNA-level expression of SDHC was abolished, as was the protein-level expression of both SDHB and SDHC. The same mechanism of disease has been described in patients with Gastrointestinal Stromal Tumors and the Carney triad[60]. Whether this mechanism of succinate dehydrogenase inactivation is prevalent in PPGLs has not been systematically investigated.

4. Imprinting

Two of the hereditary paraganglioma syndromes, PGL1 and PGL2, caused by mutations in *SDHD* and *SDHAF2* are typically inherited in a parent of origin specific manner: mutations inherited from the father but not from the mother are typically disease causing. The *SDHD*-gene was initially hypothesized to be maternally imprinted which would explain bi-allelic inactivation when the paternal copy is mutated, but this has been shown not to be the case[61]. Nevertheless, maternal imprinting has been demonstrated at an alternative promoter of a lncRNA downstream of the *SDHD* locus and it has been suggested that methylation at this locus causes changes in the chromatin conformation that in turn lead to reduced transcriptional activity at the flanking *SDHD* locus[62]. The maternally derived copy of chromosome 11 (where *SDHD* is located) is preferentially lost in these tumors and an alternative hypothesis is that one or several paternally imprinted genes on this chromosome may explain the parent-of-origin effect[63]. Potential candidates include *CDKN1C* and *H19*.

5. Mutations in epigenetic regulators

In 2015, Fishbein et al. used exome sequencing to identify *ATRX*-mutations in pheochromocytomas and paragangliomas[27]. In their cohort of 103 PPGLs 12.6% carried inactivating mutations. An association with *SDHB*-mutations was reported. These results were validated in the TCGA cohort where 5% of samples were found to carry the mutations, again in association with *SDHB*-mutations [28]. An association with aggressive disease is found in both studies. The gene product of *ATRX* dimerises with that of *DAXX* to form a chromatin remodeling complex[64] involved in the deposition of histone H3.3 proteins at the telomeres [65]. Mutations in these genes have also been described in several other tumor types including gliomas[66] and pancreatic neuroendocrine tumors[67] and are associated with poor outcome [68] in the latter. A possible effect of *ATRX*-mutations on the survival of PPGL patients has not yet been demonstrated. Inactivation of ATRX and DAXX leads to activation of an alternative lengthening of telomeres (ALT) pathway[69]. Fishbein et al. demonstrated ALT activity through telomere FISH in four out of six investigated *ATRX*-mutated tumors and two out of sixteen *ATRX*-wildtype tumors. As ALT was not detected in all cases, it is likely not the principal pathway affected by *ATRX*-inactivation in PPGLs. Notably, *ATRX*-mutations in gliomas often co-occur with mutations in the isocitrate dehydrogenase genes suggesting a possible synergistic effect of Krebs cycle disruption and *ATRX* inactivation[70].

Another histone modifier, *KMT2D*, has also been found recurrently mutated in pheochromocytomas[71], but not in abdominal paragangliomas[72]. The presence of *KMT2D*-mutations was related to larger tumor size. While the gene was not found mutated in the large TCGA cohort of 179 cases, a single missense mutation was detected by whole-exome sequencing in one of 31 tumors by Castro-Vega et al[73]. Thus the prevalence and functional relevance of the mutations remains to be elucidated in future studies. The tumor suppressor and histone methyl-transferase *SETD2* has been

described mutated in 2% of PPGLs in the TCGA cohort and is related to aggressive disease[28].

A third study using a combination of exome sequencing and RNA sequencing detected mutations in nine different chromatin modeling genes in tumors from eight individuals (*H3F3A*, *ATRX*, *EZH2*, *HIST1H1T*, *HIST4H4*, *JMJD1C*, *KDM2B*, *KMT2B* and *SETD2*, the latter nine of which were found mutated in a single individual) . Notably a postzygotic mutation in *H3F3A* (encoding histone H3.3) was detected in three tumors from a patient who had earlier been affected by giant cell tumor of bone. The same mutation was detected in another patient afflicted with the same dyad of tumors, thus characterizing a novel paraganglioma syndrome[74].

6. Histone modifications and chromatin

The focus of research into the epigenetics of PPGL so far has been on DNA methylation. The chromatin landscape of these tumors has not been well studied. This is likely due to the relative inaccessibility and high cost of methods that can be used to study the global chromatin structure. Nevertheless, some studies have been performed. Sandgren et al. used chromatin immunoprecipitation and chip technology to investigate H3K4me3 and H3K27me3 in a single malignant pheochromocytoma and related this to gene expression and copy number[75]. This enabled identification of epigenetically regulated genes that may contribute to tumor development.

In addition to inhibiting TET enzymes and prolyl hydroxylases, succinate and fumarate inhibit enzymes that regulate histone modifications such as JmjC-domain containing histone demethylases[53]. It has been demonstrated that accumulation of succinate or fumarate, as well as knock-down of *SDHB* or *SDHD* leads to increased methylation of histone H3 in several cell lines[53,76]. Increased levels of H3K9me3 have

been demonstrated in the chief cells of *SDHx*-mutated PPGLs[77]. While not yet investigated this has the potential to alter gene expression.

An interesting point for future studies is the effect of mutations in *SDHx* and *FH* on chromatin configuration. A pioneering study in gliomas demonstrated that *IDH*-mutations with resulting hypermethylation lead to disruption of the normal chromatin configuration[78]. This was shown to lead to insulator dysfunction and overexpression of the oncogene *PDGFRA*.

7. Micro RNA (miRNA)

There are very few published studied analyzing miRNA in PPGLs, most of them aiming to uncover differentialy expressed miRNAs in malignant versus benign tumors. In a pivotal study utilizing miRNA microarray analysis, Meyer et al reported over-expression of miR-438-5p (located in intronic region of *IGF2* gene) and decreased expression of miR-15a and miR-16 in malignant compared to benign pheochromocytomas [79]. Tömböl et al used the same TaqMan Human MicroRNA Cards and identified miR-1225-3p as a possible marker for recurring tumors [80]. While Patterson et al identified miR-483-5p, miR-101, and miR-183 being overexpressed in malignant tumors associated with *SDHB* mutation[81]. Differential expression of miR-210 was identified by Tsang et al. [82]. Differential expression of miR-210 and miR-183 were validated in another independent study [83]. Zong et al have shown miR-101 to be overexpressed in malignant tumors associate with SDHD mutation [84]. In general miRNA expression can collate PPGLs in distinct clusters in the same way that DNA methylation and mRNA expression can [73].

8. Conclusion

Pheochromocytomas and paragangliomas often carry epigenetic alterations. Aberrant DNA methylation has been best characterized at this point and may be important for establishing the malignant phenotype of *SDHB*-mutated tumors by altering the expression of genes involved in the regulation of the epithelial-mesenchymal transition.

Several epigenetic regulators are mutated in PPGL tumors, including *ATRX* which appears to be associated with aggressive disease. Mutations in *SDHx* and *FH* have been demonstrated to cause altered histone modifications. The functional roles of these alterations have been incompletely characterized and further work is needed to establish alterations of diagnostic and therapeutic relevance in these tumors.

8. Acknowledgements

Supported by Swedish Cancer Society and Selander Foundation. PB is a Swedish Cancer Society Investigator.

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Figure legends

Figure 1. Schematic overview of the main epigenetic alterations in the pseudohypoxia associated cluster and the kinase signaling cluster.