Review Article

DNA Methylation and Uveal Melanoma

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Abstract

Objective: The objective of the study was to summarize the role of DNA methylation in the development and metastasis of uveal melanoma (UM).
Data Sources: The relevant studies in MEDLINE were searched.
Study Selection: In this review, we performed a comprehensive literature search in MEDLINE using "uveal melanoma" AND ("DNA methylation" OR "epigenetics") for original research/review articles published before February 2018 on the relationship between DNA methylation and UM. References of the retrieved studies were also examined to search for potentially relevant papers.
Results: Previous studies on the relationship between DNA methylation and UM covered many genes including tumor suppressor genes (TSGs), cyclin-dependent kinase genes, and other genes. Among them, the TSG genes such as *RASSF1A* and *p16INK4a*, which encodes a cyclin-dependent kinase inhibitor, are relatively well-studied genes. Specifically, a high percentage of promoter methylation of *RASSF1A* was observed in UM cell lines and/or patients with UM. Promoter methylation of *RASSF1A* was also associated with the development of metastasis. Similarly, a high percentage of promoter hypermethylation of *p16INK4a* was found in UM cell lines. DNA promoter methylation can control the expression of *p16INK4a*, which affect cell growth, migration, and invasion in UM. Many other genes might also be involved in the pathogenesis of UM such as the Ras and EF-hand domain containing (*RASEF*) gene, *RAB31*, *hTERT*, embryonal fyn-associated substrate, and deleted in split-hand/split-foot 1.
Conclusions: Our review reveals the complex mechanisms underlying the tumorigenesis of UM and highlights the great needs of future studies to discover more genes/5'-C-phosphate-G-3' sites contributing to the development/metastasis of UM and explore the mechanisms through which epigenetic changes exert their function in UM.

Key words: DNA Methylation; Epigenetics; Metastasis; Uveal Melanoma

Introduction

Epigenetics is the study of heritable changes in the gene function without any change in the DNA sequence. Epigenetics includes DNA methylation, histone modification, chromatin remodeling, and noncoding RNAs (ncRNAs).[1] DNA methylation is the covalent addition of methyl groups to DNA bases, typically the cytosine of 5'-C-phosphate-G-3' (CpG) dinucleotides. It is catalyzed by methyltransferase enzymes using a S-adenosyl methionine donor and can lead to the mitotic propagation of the modified sequence with consequences for the binding of regulatory proteins such as transcription factors. DNA methylation is the most common form of epigenetics, and it changes with aging and changes in other factors such as diet and environment.[2,3] A recent study used two *Arabidopsis thaliana* mutation accumulation lines and determined that over 99.998% of the methylated regions in the genome are stably inherited across each generation indicating that DNA methylation is stably inherited by offspring and spontaneous epialleles are rare.[4]

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DNA methylation may affect the transcription of genes in two ways. First, the DNA methylation itself may physically impede the binding of transcriptional proteins to the gene. Second, and likely more importantly, methylated DNA may be bound by proteins known as methyl-CpG-binding domain proteins (MBDs). MBD proteins then recruit additional proteins to the locus, thereby forming compact, inactive chromatin, termed heterochromatin.[5]

Recent studies found that DNA methylation was involved in various age-related eye diseases such as age-related cataract,[6,7] glaucoma,[8] and age-related macular degeneration.[9,10] Uveal melanoma (UM) is a pigment cell-driving malignant neoplasm that can lead to metastasis which usually affects the liver. It is the second most common form of human melanoma and the most common primary intraocular malignant tumor in adults. The annual incidence is 6–7 cases per million.[11,12] According to the Collaborative Ocular Melanoma Study, about 50% of UM patients will develop liver metastasis within 10–15 years of enucleation.[13] Early metastasis leads to a high death rate associated with UM.[14]

Many genetic mechanisms are involved in UM. For instance, monosomy of chromosome 3 and gain of 8q are often found in UMs.[15,16] Multiple common driver mutations have also been identified in UM including *BAP1*, *EIF1AX*, *GNA11*, *GNAQ*, and *SF3B1*.[17-19] Specifically, *BAP1*, *EIF1AX*, and *SF3B1* mutations are mutually exclusive during UM progression, and *BAP1* mutations showed the most significant association with UM metastasis.[20] Meanwhile, epigenetic alteration such as changes in microRNAs and long ncRNAs also plays a role in the development and metastasis of UMs.[21,22]

Although previous studies indicated that both genetic and epigenetic alterations may affect the biology of melanoma cells by simultaneously affecting multiple proteins/pathways,[23,24] the role of epigenetics in general and DNA methylation in particular in the carcinogenesis and metastasis of UM is less well studied, compared with the genetic mechanisms involved in UM. In this article, we reviewed existing studies regarding the relationship between DNA methylation and UM. We searched MEDLINE using "uveal melanoma" AND ("DNA methylation" OR "epigenetics") for papers on the relationship between DNA methylation and UM. References of the retrieved studies were also examined to search for potentially relevant papers.

Studies of DNA Methylation in Uveal Melanoma

Previous research on the relationship between DNA methylation and UM covered multiple genes, with much attention paid to tumor suppressor genes [TSGs, Table 1]. Among them, the relatively well-studied genes are *RASSF1A* and *p16INK4a*.

Tumor suppressor genes

RASSF1A

RASSF1A, located at 3p21.3, is a TSG that is frequently hypermethylated in various human cancers.[39] A previous study found *RASSF1A* promoter methylation in 10 of the 11 (91%) UM cell lines and in 19 of 38 patients (50%) with primary UM. *RASSF1A* promoter methylation was associated with the development of metastasis.[25] Other studies also observed a high percentage of promoter methylation of *RASSF1A*.[26,27] *RASSF1A* expression is regulated by its promoter methylation.[26] These findings suggest that *RASSF1A* promoter methylation may be an early event in the progression of UM. A later study confirmed promoter methylation in UM-15 cell lines, which can be reversed after treatment with 5-aza-2'-deoxycytidine. Moreover, mice did not acquire intraocular tumors after treatment with UM-15 cells expressing exogenous *RASSF1A*.[28] These findings suggest that *RASSF1A* expression can suppress UM tumorigenesis, and epigenetic modification of gene expression might be a feasible approach for the future treatment of UM.

Ras and EF-hand domain containing gene

The Ras and EF-hand domain containing (*RASEF*) gene, also known as *RAB45* or *FLJ31614*, is located at 9q21 and is a member of the Rab GTPase family which are key regulators for membrane traffic. The protein it encodes has a distinct N-terminal EF-hand domain, a coiled-coil motif, and a C-terminal Rab domain.[40] A pervious near-genome-wide RNAi screening found *RASEF* to be hypermethylated in primary cutaneous melanomas but not nevi, indicating that it is a potential melanoma suppressor gene.[41] A study in 2008 used high-resolution melting curve and digestion analysis to screen 11 UM cell lines and 35 primary UM samples. They found that all cell lines that did not express *RASEF* and 54.5% of the primary UMs without *RASEF* expression contained hypermethylation in the promoter region, whereas those with *RASEF* expression contained an unmethylated promoter. Promoter methylation of *RASEF* was associated with increased risk of death due to metastasis, and patients with a homozygous genotype and methylation of *RASEF* had a significantly higher risk of metastasis than those with a heterozygous genotype and no methylation.[29] These results suggest that homozygosity of *RASEF* in combination with methylations might be an important mechanism by which *RASEF* led to UM.

BRCA1-associated protein-1

BRCA1-associated protein-1 (*BAP1*), located at 3p21, encodes a deubiquitinating enzyme involved in the removal of ubiquitin from proteins.[42] A previous study found inactivating somatic mutations in more than 80% of metastasizing tumors, implicating the involvement of loss of *BAP1* in UM metastasis.[17] A recent comprehensive multiplatform analysis of 80 UM identified four molecularly distinct, clinically relevant subtypes: two associated with poor-prognosis monosomy 3 (M3) and two with better-prognosis disomy 3 (D3).[32] For the poor-prognosis

Table 1: Summary of major findings from previous literature

Category	Genes	Chromosome	Function	References	Testing method	Sample	Major findings
Tumor suppressor genes	*RASSF1A*	3p21.3	Ras-associated domain family	[25]	PCR in combination with melting curve analysis, sequencing, and restriction enzyme analysis	11 cell lines and 38 UMs	DNA methylation in 91% UM cell lines, and in 50% primary Ums, promoter methylation associated with UM metastasis
				[26]	Methylation-specific PCR	42 UMs and 8 UM cell lines (7 primary and 1 metastatic UM)	Promoter methylation of *RASSF1A* was detected in 35 of 42 tumors (83%)
				[27]	Methylation-specific PCR	20 UM samples	Methylation rate of *RASSF1A* was 70%
				[28]	QRT-PCR	UM-15 clone	Lack of *RASSF1A* expression and full methylation of the *RASSF1A* promoter region in the UM-15 cell line
	RASEF	9q21	Ras pathway	[29]	HRM and digestion analysis, RT-PCR	11 UM cell lines and 35 primary UMs	All the five *RASEF*-expressing cell lines contained an unmethylated promoter while hypermethylation of all CpGs within the amplicon was present in all the six cell lines that lacked *RASEF* expression. There was a correlation between methylation and expression of *RASEF* in the primary tumor samples, but not as obvious as in the cell lines
	ITGA7/ NDRG2/ PITX2	12q13.2/14q11.2/4q25	Tumorigenesis	[30]	Bisulfite sequencing validation	63 cases of human UM	Promoter hypermethylation was extensively observed in these genes (85% methylated samples) in UM
	RAB31	18p11.22	Ras oncogene family	[31]	Methylation-specific PCR	67 UMs	Differential methylation between normal uvea and UM
	BAP1	3p21.1	Ubiquitin C-terminal hydrolases	[32]	Illumina infinium HM450 array	80 UMs	M3 developed initially followed by alterations of *BAP1*, which has distinct global DNA methylation from that observed in D3
Gylin-dependent kinase	*p16INK4a*	9p21	Stabilizer of the tumor suppressor protein	[33]	Methylation-specific PCR	12 UM cell lines and 22 UMs	Promoter is hypermethylated in 6 of 12 UM cell lines and in 7 of 22 primary UMs

Contd...

Table 1: Contd...

Category	Genes	Chromosome	Function	References	Testing method	Sample	Major findings
Other genes	*TIMP3*	22q12.3	Extracellular matrix degradation	[34]	Microarray analysis and demethylation test	UM cell lines and 2 metastatic samples	5-fold decreased expression of *TIMP3* in the metastatic cell lines
	PRAME	22q11.22	Encodes an antigen preferably expressed in human melanoma	[35]	Methylation probes in Methyl450K array	678 UM patients	12 CpG sites within and near the *PRAME* promoter region were hypomethylated in PRAME+ tumors
	hTERT	5p15.33	Telomerase reverse transcriptase	[36]	Methylation-sensitive single-strand conformation analysis and dot-blot assay	23 primary UMs	hTERT promoter methylation was found with a relatively high frequency (52%)
	EFS	14q11.2	CAS protein family	[37]	PCR	16 UMs	Full methylation of the *EFS* CpG island in 8 (50%), no methylation in 5 (31%), and partial methylation in 3 (19%) UMs
	DSS1	7q21.3–q22.1	DSB repair	[38]	Methylation-specific PCR	130 cutaneous melanomas, 64 UMs, 82 mucosal melanomas, and 75 SCC samples	There was an inverse correlation between *DSS1* expression and methylation status of the promoter

CpG: C–phosphate–G; HRM: High-resolution melting-curve; PCR: Polymerase chain reaction; QRT-PCR: Quantitative real-time polymerase chain reaction; RT-PCR: Real-time polymerase chain reaction; SCC: Squamous cell carcinomas; UMs: Uveal melanomas; CAS: Cellular apoptosis susceptibility; DSB: DNA double-strand break; *RASEF*: Ras and EF-hand domain containing; *EFS*: Embryonal fyn-associated substrate; *DSS1*: Deleted in Split-Hand/Split-Foot 1; *PRAME*: Melanoma antigen preferentially expressed in tumors; *TIMP3*: Tissue inhibitor of metalloproteinases 3; *BAP1*: BRCA1 associated protein-1; *ITGA7*: Integrin alpha 7 subunit; *NDRG2*: N-myc downstream-regulated gene 2; *PITX2*: Paired-like homeodomain 2.

UM, M3 developed initially followed by alterations of *BAP1* which has distinct global DNA methylation from that observed in D3. For the better-prognosis D3, those with *EIF1AX* mutations had different DNA methylation patterns compared with those with *SF3B1*/*SRFR2* mutations. These findings suggest that *BAP1* mutations may lead to metastasis-prone DNA methylation states.

Other tumor suppressor genes

An early study investigated the methylation status of several TSGs including the fragile histidine triad (*FHIT*), von Hippel-Lindau (*VHL*), β-catenin (*CTNNB1*), activated leukocyte cell adhesion molecule (*ALCAM*), retinoic acid receptor-β2 (*RARB*), the CpG-rich regions 5' of the E-cadherin (*CDH1*), p16/cyclin-dependent kinase inhibitor 2A (*CDKN2A*), retinoblastoma, and *SNRPN* and *D15S63* loci.[43] Out of the 40 UMs that were examined, three exhibited hypermethylation in *SNRPN* and *D15S63*, three had methylated alleles in *RARB*, and three in *CDKN2A*. Although loss of one copy of chromosome 3 (M3) is found in approximately half of UMs and is associated with metastasis, no methylation was detected in the CpGs islands of TSGs located on chromosome 3 including *FHIT*, *VHL*, *CTNNB1*, and *ALCAM*. Because *VHL* and *FHIT* are known to be inactivated by promoter methylation in various cancer entities, these findings imply that epigenetic changes in these genes are unlikely to play a pivotal role in the tumorigenesis of UM.

Another recent study examined the genome-wide methylome of 63 cases of human UM and 12 nonpathological choroid/retinal pigment epithelium (RPE)/ciliary body tissues as controls and identified 1841 CpGs that were differentially methylated. Of them, 45% were hypermethylated and 55% were hypomethylated. Gene Ontology annotations revealed enrichment of cell differentiation, cell development, and signal regulation. Special attention was given to integrin subunit alpha 7 (*ITGA7*), N-myc downstream-regulated gene 2 (*NDRG2*), and paired-like homeodomain transcription factor 2 (*PITX2*) due to their roles in tumorigenesis. The extensive promoter hypermethylation was also observed in an independent cohort of 67 UM samples.[30] Interestingly, the study found that out of the 12 genes that were previously reported to be associated with metastatic risk of UM.[31] Only *RAB31*, a member of the RAS oncogene family, showed differential methylation between normal uvea and UM. Moreover, its promoter hypermethylation is associated with increased overall survival.

Another study of 23 primary UMs examined CpG promoter methylation of several cancer-related genes including

p16INK4a, Tissue inhibitor of metalloproteinases 3 (*TIMP3*), *RASSF1*, *RARB*, *FHIT*, *hTERT*, and *APC*. A relatively high frequency of CpG promoter methylation (52%) was found only in hTERT, with low frequency of methylation (<15%) in *p16INK4a*, *TIMP3*, *RASFF1*, and *RARB*, and no methylation in *FHIT* and *APC*.[36] Given the presence of promoter methylation of *APC*, *RASSF1*, and *RARB* in cutaneous melanoma, these findings suggest that cutaneous and UM likely undergo different epigenetic changes.

Cyclin-dependent kinase gene

The *p16INK4a* is a cyclin-dependent kinase inhibitor, whose gene is located on 9p21. Previous studies have found that loss of *p16INK4a* expression in patients with progressing melanoma is associated with increased tumor cell proliferation and decreased patient survival.[44,45] An early methylation-specific polymerase chain reaction analysis of *p16INK4a* showed that both primary UM and UM cell lines had a high percentage of promoter hypermethylation of *p16INK4a* (32% and 50%, respectively). The promoter hypermethylation decreased its expression. UM patients with promoter hypermethylation also had a higher death rate due to metastasis, compared with those with nonmethylated primary tumor (71% vs. 13%).[33] The study also reported that loss of *p16INK4a* expression could be reversed by 5-aza-2'-deoxycytidine, a demethylating drug. A more recent study reported no *p16INK4a* mRNA in 50% of UMs, and aberrant methylation in homozygosis was found in all the UMs that did not express *p16INK4a* mRNA, while no methylation was detected in the UMs that exhibited *p16INK4a* expression.[38,46] The study provided convincing evidence that promoter methylation of *p16INK4a*-controlled expression of the gene, which can drastically affect cell growth, migration, and invasion in UM. These findings indicated that promoter methylation of *p16INK4a* accompanied by loss of its expression is common in UM, implying a potential role that this gene might play in the tumorigenesis of UM. Another study of 23 primary UMs, however, found only one case (4%) with promoter methylation in *p16INK4a*.[36] The inconsistency in findings might be due to the different techniques used for the detection of methylation status. As revealed,[47] often less than 10% of tumor DNA are methylated. This low percentage might help explain the failure to detect methylation using the dot-blot assay technique in the later study.

Other genes

Tissue inhibitor of metalloproteinases 3

TIMP3, located at 22q12, belongs to the TIMP gene family which encodes a group of peptidases involved in the degradation of the extracellular matrix.[48] *TIMP3* is generally expressed by the RPE,[49] and mutations in *TIMP3* can cause hereditary blindness.[50] An early study found that, compared with a normal choroidal melanocyte cell culture, the mRNA level of *TIMP3* decreased by about 80% in the primary UM cell line and by more than 95% in the metastatic UM cell lines,[34] and the decrease was regulated by promoter methylation of *TIMP3*. These findings suggest that methylation of *TIMP3* likely is involved in the development of UM.

PRAME

Melanoma antigen preferentially expressed in tumors (*PRAME*), located at 22q11, is a gene that encodes an antigen preferably expressed in human melanoma, with no or minimal expression in normal tissues except testis and endometrium.[51] A study of 678 UM patients found that 12 CpG sites within and near the *PRAME* promoter region were hypomethylated in PRAME+ tumors, compared to PRAME− tumors. A significant correlation was observed between the level of hypomethylation of all the 12 CpGs and the level of mRNA expression.[35] Aberrantly hypomethylation of *PRAME* is associated with increased UM metastasis. Moreover, *PRAME*-specific T-cells reacted against *PRAME*-positive UM cell lines.[52] These findings suggested that *PRAME* hypomethylation might be involved in the tumorigenesis of UM and its metastasis, and that *PRAME*-directed immunotherapy might be an option for certain patients with metastatic UMs.

Embryonal fyn-associated substrate

Embryonal fyn-associated substrate (*EFS*) is a member of cellular apoptosis susceptibility protein family and is now being recognized as playing important roles in multiple immune cell processes.[53] Full or partial methylation of *EFS* CpG island was observed in 81% of UMs. *EFS* methylation was only observed in UM with metastases and is associated with a higher risk of metastatic progression. EFS methylation is biallelic and tissue specific, with full methylation in peripheral blood cells, partial methylation in cultured melanocytes from the uveal tract, and no methylation in sperm, kidney, and brain.[37] These findings suggest that there are methylated and unmethylated precursor cells, and *EFS* methylation in UM may depend on the type of precursor cells from which the tumor originated.

Deleted in split-hand/split-foot 1

Deleted in split-hand/split-foot 1 (*DSS1*) is a gene involved in DNA double-strand break (DSB) repair and may also be involved in cell differentiation, proliferation, and transformation. A recent study found that *DSS1* hypomethylation led to elevated mRNA expression, which is associated with metastasis of UM, shorter overall survival (average 12.5 months vs. 18.5 months), and shorter disease-free survival (11.1 months vs. 22.3 months) of patients with UM. These findings suggested a potentially important role that epigenetic changes of *DSS1* could play in the tumorigenesis of UM.[38]

Conclusions

DNA methylation has been proved to be associated with the development, progression, and metastasis of UM. Among the many potential genes contributing to UM, *RASSF1A*, a TSG, and *p16INK4a*, which encodes a cyclin-dependent kinase, have been relatively well studied, when compared with the other genes. More studies are also needed on the

potential role of DNA methylation of other genes which harbor major identified mutations for UM such as *GNA11*, *GNAQ*, *EIF1AX*, and *SF3B1*. However, it is still very difficult to build a comprehensive network for UM to elucidate all the genetics and epigenetic mechanisms including DNA methylation. Early diagnosis of this malignant tumor plays a crucial role in the successful prevention and treatment of UM. With increasing knowledge of epigenetics, the study of DNA methylation will help in finding biomarkers for early diagnosis, classification, and prognosis. 5-aza-2'-deoxycytidine and zebularine have been shown to be effective in the treatment of hematologic disorders[54] as well as in the *in vitro* model of cataract.[55,56] Such medicine that focuses on DNA methyltransferase might shed a light on the treatment of this malignancy. However, more in-depth understanding of the mechanisms linking DNA methylation with UM is greatly needed to develop drugs targeting DNA methylation.

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Conflicts of interest

There are no conflicts of interest.

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DNA甲基化与葡萄膜黑色素瘤

摘要

目的：总结DNA甲基化在葡萄膜黑色素瘤（UM）的发生和转移中的作用。
数据来源：在MEDLINE上对相关文献进行检索。
研究选择：在本综述中，使用“葡萄膜黑色素瘤”和“DNA甲基化”或“表观遗传学”在MEDLINE中进行了详尽的文献检索，选择在2018年2月前发表的关于DNA甲基化与UM的原创研究和综述类文章。此外，我们也对检索到的文献中的参考文献进行了进一步筛选，以尽可能涵盖所有相关文献。
结果：先前关于DNA甲基化与UM之间关系的研究涉及很多基因，包括抑癌基因（TSG），细胞周期蛋白依赖性激酶基因及其他基因。其中，对于TSG中的RASSF1A基因和以及编码细胞周期蛋白依赖性激酶抑制剂的 p16INK4a基因的研究较为充分。特别是UM细胞系以及UM患者中RASSF1A基因有很高比例发生启动子的甲基化；同时RASSF1A启动子的甲基化与转移的发生相关。同样，UM细胞系中也有很高比例出现了启动子的甲基化。DNA启动子的甲基化能够控制p16INK4a的表达，进而影响UM中细胞的生长、迁移和侵袭。UM的发病机制中可能还涉及很多其他基因，如RASEF、RAB31、hTERT、EFS，以及DSS1。
结论：本综述揭示了UM在肿瘤的发生过程中可能涉及的复杂机制。今后的研究需要发现更多与UM的发生和转移相关的基因以及5’-C-磷酸-G-3’位点，并探索表观遗传学在UM中的作用。