REVIEW ARTICLE

Role of Notch signalling pathway in cancer and its association with DNA methylation

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Abstract

The Notch signalling pathway is an evolutionarily conserved cell signalling pathway involved in the development of organisms as diverse as humans and fruit flies. It plays a pivotal role in cell fate determination. Dysregulated Notch signalling is oncogenic, inhibits apoptosis and promotes cell survival. Abnormal Notch signalling is seen in many cancers like T-cell acute lymphoblastic leukaemia, acute myeloid leukaemia and cancers of the breast, cervix, colon, pancreas, skin and brain. Inhibition of Notch signalling leads to growth arrest and differentiation in those cells in which Notch pathway is activated and this represents a new target for cancer therapy. Cancer develops from genome defects, including both genetic and epigenetic alterations. Epigenetics deals with heritable changes in gene function that occur without a change in the DNA sequence. Among various epigenetic alterations such as acetylation, phosphorylation, ubiquitylation and sumoylation, promoter region methylation is considered as an important component in cancer development. Epigenetic alterations can be used as biomarkers in screening, detection, diagnosis, staging and risk stratification of various cancers. DNA methylation can be therapeutically reversed and demethylating drugs have proven to be promising in cancer treatment. This review focusses on the methylation status of genes in Notch signalling pathway from various cancers and how this epigenetic alteration can be used as a biomarker for cancer diagnosis and subsequent treatment.

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Introduction

The Notch gene was first discovered in *Drosophila melanogaster*, with an adult phenotype consisting of 'notches' at the wing margin by Thomas Hunt Morgan in 1917. The pathway comprises of ligands, receptors, transcriptional complex components and downstream genes. In mammals, there are four different Notch receptors and five ligands. Notch signalling is triggered by direct interaction of receptors with ligands expressed on neighbouring cells. This releases the Notch intracellular domain (NICD) from the membrane after cleavage by gamma-secretase. NICD then translocates into the nucleus and associates with transcription factors, which leads to the expression of Notch target genes.

Aberrant Notch signalling is associated with several human diseases including cancers. Dysregulated Notch signalling contributes to tumour development by altering the developmental state of a cell and consequently maintaining the cells in a proliferative or undifferentiated fate. Thus, Notch signalling plays a crucial role in tumour development by causing cells to adopt a proliferative cell fate. Inhibitors of Notch signalling like gamma-secretase inhibitors (GSIs) are widely used *in vitro* and *in vivo* for cancer therapy (Shih and Wang 2007).

Epigenetics refers to stable and heritable alteration in gene expression potential, without any change in the DNA sequence of the gene. Recent studies have shown that epigenetic events play a significant role in the development and progression of cancer, viral infections, genomic imprinting, developmental abnormalities, mental health and X-chromosome inactivation. DNA methylation is one of the most commonly occurring epigenetic events, in which there is an addition of a methyl (CH_3) group to the cytosine ring at the carbon 5 position in the sequence 5′CpG3′ by enzyme DNA methyltransferase. Hypermethylation in the promoter region leads to reduced gene expression by interfering with the binding of specific transcription factors to their recognition sites or by binding of transcriptional repressors specific for the methylated DNA sequence (Razin and Cedar 1991).

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Cancer cells show disrupted methylation patterns in their DNA. Repeated DNA sequences, such as long interspersed nuclear elements are involved in hypomethylation, whereas CpG islands are involved in hypermethylation. In cancer, hypermethylation is more common compared to hypomethylation. Hypermethylation in cancer cells is mostly seen in genes that are involved in cell cycle regulation ($p16^{INK4a}$, $p15^{INK4a}$, *Rb*, p^{14ARF}), genes associated with DNA repair (*BRCA1*, *MGMT*), apoptosis (*DAPK*, *TMS1*), drug resistance, detoxification, differentiation, angiogenesis and metastasis. Hypomethylation contributes to oncogenesis by activation of oncogenes or by activation of latent retrotransposons such as long interspersed nuclear elements. Hypomethylation of retrotransposons or mobile DNA can cause cancer due to disruption of expression of the adjacent gene (Das and Singal 2004).

Tumour-specific methylation changes in different genes have shown to be effective in cancer diagnosis, prognosis and therapeutics. Unlike the traditional methods of diagnosis such as histopathology, immunohistochemistry, etc., molecular markers can further subclassify the tumours and provide information in response to chemotherapeutic agents and survival. As methylation changes often occur during the initial stages of tumourigenesis, it can be used as a biomarker in early diagnosis of cancer. Experiments have shown that DNA methylation is reversible and demethylating drugs can reverse the silencing of genes resulting from methylation, thus making demethylation a therapeutic target (Mossman *et al.* 2010). Drugs used for demethylation are found to cause cell death by obstructing DNA synthesis or induce structural instability and DNA damage or bind to DNA methyltransferase enzyme, making it inactive. However, further research is needed to check the feasibility of using methylation inhibitors in combination with histone deacetylases and conventional chemotherapeutic agents in the treatment of cancer. By documenting exact methylation profiles of tumours and designing drugs that target these sequences, a more effective and promising class of cancer treatments can be made available.

Notch signalling pathway

The Notch signalling pathway is an evolutionarily conserved cell signalling pathway involved in the development of invertebrate and vertebrate species. It is necessary for communication between adjacent cells, involving regulation of gene expression that controls multiple cell differentiation during embryonic and adult life (figure 1). Notch pathway mediates signalling between adjacent cells by which cell fate decisions are regulated in neuronal, immune, cardiac and endocrine development. In mammals, there are four different Notch receptors and five ligands, which are referred to as Notch 1, Notch 2, Notch 3 and Notch 4; Delta-like 1, Delta-like 3, Delta-like 4, Jagged 1 and Jagged 2. The Notch receptors span the cell membrane, with intracellular and extracellular domain and the Notch ligands are members of the DSL (Delta/Serrate/LAG-2) family of single-pass transmembrane proteins. Ligand proteins on the adjacent cell membrane binding to the extracellular domain of Notch receptor induce proteolytic cleavage and release the intracellular domain, which enters the cell nucleus and engage other DNA-binding proteins, thus regulating gene expression.

After translation, chaperone *O*-fucosyltransferase (O-Fut) fucosylates the Notch protein, which is essential for the production of a functional receptor. Later, glycosyltransferase activity of Fringe extends fucose, altering the ability of specific ligands to activate Notch. The mature receptor undergoes proteolytic cleavage by protein convertases

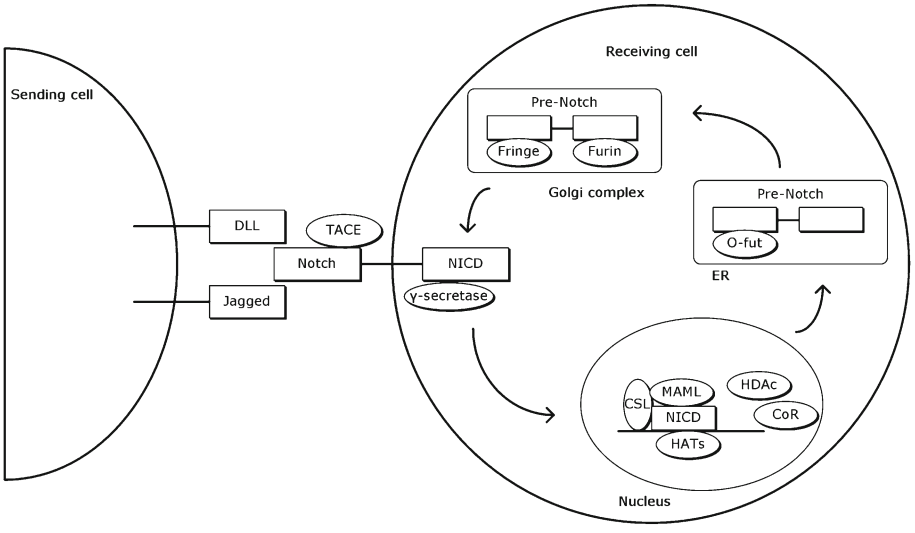

Figure 1. Schematic representation of Notch signalling pathway.

(PC5; Furin) at site 1 (S1) in the Golgi complex and then the heterodimer is targeted to the cell surface held together by noncovalent interactions (Ilagan and Kopan 2007). Once the Notch extracellular domain interacts with a ligand, an ADAM-family (a disintegrin and metalloproteinase) metalloprotease called tumour necrosis factor alpha converting enzyme (TACE) cleaves the Notch protein just outside the membrane at site 2 (S2) (Brou *et al.* 2000). This releases the extracellular portion of Notch, which is endocytosed by the ligand-expressing cell and is recycled/degraded within the cell. After this cleavage, an enzyme called gamma-secretase cleaves remaining portion of the Notch protein just on the inner side of the cell membrane at site 3 (S3), releasing the NICD. NICD translocates to the nucleus, and forms a complex with the DNA-binding protein CSL (CBF1/RBPjκ/Su(H)/Lag-1), displacing a histone deacetylase (HDAc) corepressor (CoR) complex from CSL. In the absence of NICD, transcription of target genes is repressed by DNA-binding protein CSL in association with ubiquitous corepressor (CoR) proteins and HDAcs. MAML1 (mastermind-like proteins) and histone acetyltransferases (HATs) bind to the NICD–CSL complex, leading to the transcription of Notch target genes.

Transcription regulators like DTX1, FOS, HES1, FOSL1, HEY1, NFKB1, NFKB2, PPARG, STAT6 and NR4A2, encoded by Notch pathway target genes, modulate cell fate affecting the function of tissue-specific basic helix-loop-helix (HES) gene family or through other molecular targets, such as NF-kappaB. These in turn influence lineage commitment by regulating expression of tissue-specific transcription factors. Other potential Notch targets include $p21^{WAF1/Cip1}$, cyclin D1, homocysteine-induced endoplasmic reticulum protein (HERP) and mitogen-activated protein kinase phosphatase LIP-1 (Wu *et al.* 2002); apoptosis genes such as *CDKN1A*, *CFLAR* (*CASH*), *IL2RA*; cell cycle regulators like *CCND1*, *CDKN1A*, *IL2RA*; genes involved in cell proliferation like *CDKN1A*, *ERBB2*, *FOSL1*, *IL2RA*; genes regulating cell differentiation which includes *DTX1*, *PPARG*; genes involved in neurogenesis such as *HES1*, *HEY1*; there are also few genes with unspecified functions like *CD44*, *CHUK*, *IFNG*, *IL17B*, *KRT1*, *LOR*, *MAP2K7*, *PDPK1*, *PTCRA*. Sel10 and Su(Dx) (suppressor of Deltex), members of E3 ubiquitin ligase family control metabolism of NICD in the nucleus by phosphorylation and ubiquitination. NICD degradation resets the cell and triggers next round of Notch signalling (Kopan 2002).

Notch signalling pathway and cancer

Apart from having critical roles in differentiation, proliferation and survival, the Notch signalling pathway is also found to be involved in different cancer types. However, its components act as both oncogenes and tumour suppressor genes (TSG) in a variety of malignancies depending on the type of tissue. Notch signalling pathway may thus present novel therapeutic targets in the treatment of cancer (figure 2).

Notch as oncogene

Dysregulated Notch signalling pathway causes a variety of disorders, including human malignancies (table 1). It is shown that Notch signalling is involved in the maintenance of glioma stem cells (GSC). Chigurupati *et al.* (2010) showed elevated *NOTCH1* expression in glioblastoma contributed by hypoxia. Notch signalling is found to play an important role in different forms of haematologic malignancies including T-cell lymphoblastic leukaemia (Aster *et al.* 2011). Also, *NOTCH1* and its downstream target genes are activated in stem cell leukeamia-lymphoma syndrome (SCLL). Notch signalling pathway activation was seen in hepatocellular carcinoma caused due to a protein NS3 (non-structural protein 3) from hepatitis C virus (Iwai *et al.* 2011). Recent evidence suggests that *NOTCH3* is overexpressed in human lung cancer cell lines. Inhibition of Notch signalling by gamma-secretase inhibitors (MRK-003) decreased proliferation *in vitro* (Osanyingbemi-Obidi *et al.* 2011). Efforts are being made to block tumour growth by targeting Notch pathway in most cancer types (Kuhnert *et al.* 2011; Gu *et al.* 2012).

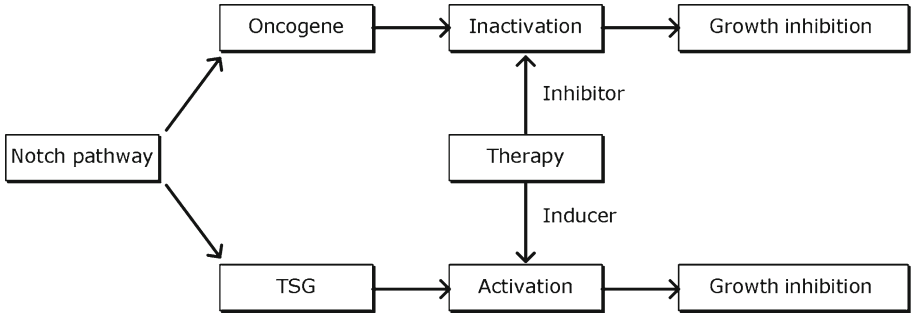

Figure 2. Duality of Notch function in cancer: Notch genes exert both oncogenic and tumour suppressor role in various malignancies. This has an important implication in cancer therapy. Notch inhibitors or inducers can be used accordingly in cancer therapy imparting growth inhibitory functions.

Table 1. Oncogenic and tumour suppressor role of Notch signalling pathway in the development of cancer.

Role of Notch signalling	Cancer type	Gene	References
Oncogene	Breast cancer	*NOTCH1*, *NOTCH3*, *NOTCH4*, *JAG1*, *DLL4*	Parr *et al.* (2004) Zardawi *et al.* (2010) Harrison *et al.* (2010) Yamaguchi *et al*. (2008) Cohen *et al.* (2010) Jubb *et al.* (2010) Mittal *et al.* (2009)
	Cervical cancer	*NOTCH2*, *NOTCH3*	Talora *et al.* (2002) Yeasmin *et al.* (2010)
	Colorectal cancer	*NOTCH1*, *NOTCH3*, *DLL4*, *JAG1*, *JAG2*	Jubb *et al.* (2009) Zhang *et al.* (2010) Serafin *et al.* (2011) Reedijk *et al.* (2008) Rodilla *et al.* (2009) Meng *et al.* (2009)
	Ovarian cancer	*NOTCH1*, *NOTCH3*, *DLL4*, *JAG1*	Rose *et al.* (2010) McAuliffe *et al.* (2012) Hu *et al.* (2011) Choi *et al.* (2008) Steg *et al.* (2011)
	Gastric cancer	*NOTCH1*, *NOTCH2*, *JAG1*	Hsu *et al*. (2012) Sun *et al.* (2011) Yeh *et al*. (2009) Tseng *et al.* (2012)
Tumour suppressor gene	Breast cancer	*NOTCH2*	Parr *et al.* (2004)
	Cervical cancer	*NOTCH1*	Talora *et al.* (2002)
	Colorectal cancer	*NOTCH1*, *NOTCH2*	Kim *et al.* (2012) Chu *et al.* (2009)
	Pancreatic cancer	*NOTCH1*	Kunnimalaiyaan *et al.* (2005)
	B-cell malignancies	*NOTCH1*, *NOTCH2*, *NOTCH3*, *NOTCH4*, *JAG1*, *JAG2*	Zweidler-McKay *et al.* (2005)
	Prostate cancer	*NOTCH1*, *JAG1*	Shou *et al.* (2001) Zhang *et al*. (2006)
	Myeloblastic leukaemia	*DLL1*, *DLL4*	Tohda *et al.* (2003)
	Hepatocellular carcinoma	*NOTCH1*	Qi *et al.* (2003)
	Thyroid cancer	*NOTCH1*	Kunnimalaiyaan *et al.* (2006) Ferretti *et al.* (2008)
	Brain tumour	*NOTCH1*	Fan *et al.* (2004)
	Skin cancer	*NOTCH1*	Nicolas *et al.* (2003)

Inhibition of Notch pathway by GSI depleted stem-like cancer cell proliferation and increased apoptosis in glioblastoma multiforme (GBM) (Chen *et al.* 2010; Fan *et al.* 2010). Glioma cell lines showed decreased cell growth, enhanced cell cycle arrest and apoptosis by knockdown of *NOTCH1* gene (Zhao *et al.* 2010). In prostate cancer cells, *NOTCH1* gene silencing using small interfering RNA-induced apoptosis by downregulating antiapoptotic protein Bcl-2, and upregulating proapoptotic protein Bax, ultimately inhibit proliferation (Ye *et al.* 2012). Inhibition of Notch pathway by gamma-secretase inhibitors in T-cell acute lymphoblastic leukaemia cell lines showed growth inhibition and cell cycle exit (Rao *et al*. 2009). Further, Ren and Cowell (2011) have shown that inhibition of Notch signalling by gamma-secretase inhibitors and by shRNA targeting Notch 1 and its cofactors mastermind-like 1 and c-promoter-binding factor 1 (MAML1 and CBF1) delayed leukaemogenesis *in vivo*. Inhibition of overexpressed *NOTCH3*, *NOTCH4* and *JAG1* in pancreatic cancer tissue using gamma-secretase inhibitors resulted in increased cell death and tumour suppression (Vo *et al.* 2011).

Notch as tumour suppressor

Notch pathway genes can act as tumour suppressors or oncogenes depending on the cellular context (table 1). Inactivation of Notch signalling in mouse haematopoietic stem cells (HSCs) resulted in an aberrant accumulation of granulocyte/monocyte progenitors (GMPs), extramedullary haematopoiesis and induced chronic myelomonocytic leukaemia-like disease. The study also identified a novel role for Notch signalling during early haematopoietic stem cell differentiation and suggested that the Notch pathway

can play both tumour inducive and suppressive roles within the same tissue (Klinakis *et al.* 2011). Overexpression of *NOTCH1* and *NOTCH2* in small cell lung cancer cell lines caused growth arrest, associated with a G_1 cell cycle blockage. Upregulation of $p21^{waf1/cip1}$ and $p27^{kip1}$ were also observed in concert with the cell cycle changes (Sriuranpong *et al.* 2001). Activation of Notch 1 signalling in cervical cancer cells induced apoptosis, cell cycle arrest and tumour suppression (Franko-Tobin *et al.* 2012). Dotto (2008) described that the Notch pathway genes function as tumour suppressors, by loss of function in keratinocytes. In hepatocellular carcinoma, activation of the Notch pathway via E2F transcription factors, leads to tumour regression (Viatour *et al.* 2011). In head and neck squamous cell carcinoma, 40% of the 28 mutations in *NOTCH1* were found to truncate the gene product showing tumour suppressor role of the gene (Agrawal *et al.* 2011). Additional study showed mutations in *NOTCH1* gene resulting in its dysregulation leading to head and neck squamous cell carcinoma (Stransky *et al.* 2011). Yet another study by Kunnimalaiyaan and Chen (2007) showed activation of Notch 1, which is usually absent in neuroendocrine tumours, reduced tumour growth *in vitro*.

Methylation of Notch pathway genes in cancer

DNA methylation is an epigenetic way of inheritance without any alterations in the DNA sequence. It involves methylation of cytosine nucleotide usually adjacent to a guanine nucleotide (CpG islands) by DNA methyltransferase (DNMT) enzyme, forming 5-methylcytosine in the upstream region of promoter sequence. DNA methylation is found to be responsible for regulation of imprinted genes, X chromosome inactivation, tumour suppressor gene silencing in cancer, control of gene expression, chromosomal integrity and recombinational events.

The role of DNA methylation in development of cancer is one of the hottest and widely studied areas in cancer biology. Global and local distribution of 5-methylcytosines have been studied in normal cells and changes in this DNA methylation pattern seen in cancer cells have been used as biomarkers for diagnosis and treatment (figure 3). Apart from using DNA methylation status as a biomarker for cancer diagnosis, it can also be used to identify different cancer types, aid in tracing the primary origin of metastatic tumours, detect the stage of cancer and assess the risk of progression to malignancy. Monitoring DNA methylation pattern after treatment can provide evidence of treatment efficacy and detect cancer recurrence. Thus DNA methylation is an important area in the field of cancer biology.

Not much is known about the methylation status of Notch pathway genes in cancer. Both Notch gene hypermethylation and hypomethylation have been reported in some human cancers. Lack of Notch 1 ligand *DLL1* expression in gastric cancer cell lines was associated with promoter hypermethylation resulting in decreased *NOTCH1* expression. This indicates that Notch 1 activity in gastric cancer is controlled by epigenetic silencing of *DLL1* (Piazzi *et al.* 2011). Comparison of methylation pattern of certain genes between tumour and surrounding tissue from hepatocellular carcinoma patients, showed reduced methylation of *NOTCH4* gene promoter in tumour compared to surrounding tissue (Hernandez-Vargas *et al.* 2010). Notch pathway genes

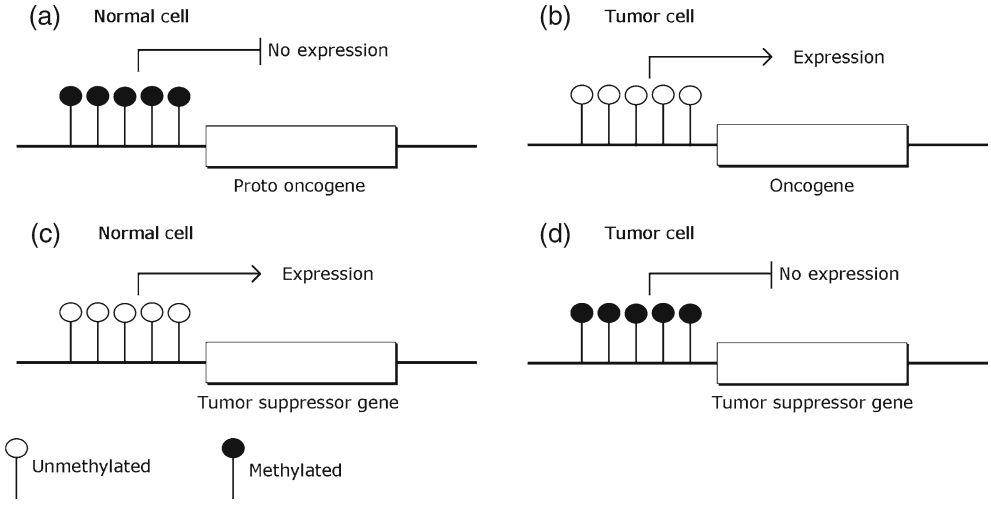

Figure 3. Methylation status of oncogene and tumour suppressor gene in normal and tumour cells: (a) DNA hypermethylation resulting in transcriptional silencing of proto-oncogene in normal cells. (b) DNA hypomethylation causing activation of oncogenes in tumour cells. (c) Actively transcribed tumour suppressor gene associated with DNA hypomethylation in normal cells. (d) DNA hypermethylation of tumour suppressor gene leading to gene silencing in tumour cells.

such as *DLL1*, *HEY1*, *DTX1*, *HDAC1*, *NOTCH2* and *JAG1* had low expression levels but were not found to be methylated in hepatoblastoma (Aktas *et al.* 2010). *JAG2* is overexpressed in multiple myeloma cells. The methylation status of *JAG2* promoter was studied to correlate methylation pattern with its expression level. The promoter region of *JAG2* gene was hypomethylated in malignant cells from multiple myeloma patients and cell lines (Houde *et al.* 2004; Ghoshal *et al.* 2009). Though the promoter region was found to be hypomethylated, it could not be responsible for its upregulation as even nonexpressing cells showed hypomethylated *JAG2* promoter sequence. These results suggest that DNA methylation does not play a role in *JAG2* gene expression level in multiple myeloma cell lines (Ghoshal *et al.* 2009). Due to lack of sufficient evidence correlating DNA methylation and its effects on Notch gene expression, at present there are no biomarkers developed using methylated Notch gene promoter for cancer diagnosis.

Demethylation as cancer therapy

Abnormal DNA CpG island hypermethylation is transcriptionally repressive resulting in silencing of tumour suppressor genes which in turn leads to the development of various human cancers. Unlike genetic changes that occur in the genome, epigenetic changes are potentially reversible. Drugs that reverse DNA methylation can decrease tumour cell growth by reactivating epigenetically silenced tumour suppressor genes. At present, demethylation is an attractive emerging prevention and therapy target for cancer.

Reagents targeting enzymes involved in epigenetic modifications have the potential to reverse or inhibit epigenetic changes in cancer cells. Demethylating agents such as 5-aza-2′-deoxycytidine (5-Aza C) were being used as chemotherapy agents even before promoter methylation was known to be involved in cancer development (Baylin *et al.* 2001). DNA methyltransferase inhibitors 5-aza-2′-deoxycytidine and zebularine have dual action of angiostatic activity and inhibitory effects on tumour cells by reactivating the growth-inhibiting genes which are suppressed in tumour-conditioned endothelial cells (Hellebrekers *et al.* 2006). 5-aza-2′-deoxycytidine treatment activated more genes in human bladder tumour cell line (T24) than in nontumourigenic human fibroblast cells (LD419) (Liang *et al.* 2002).

Dietary polyphenols, such as (−)-epigallocatechin 3-gallate (EGCG) from green tea and genistein from soybean inhibit DNA methyltransferases *in vitro* and also induce demethylation of the CpG islands in the promoters of methylation-silenced genes such as $p16^{INK4a}$, retinoic acid receptor β, O^6-methylguanine methyltransferase, human mutL homolog 1 and glutathione *S*-transferase-π in human oesophageal, colon, prostate and mammary cancer cell lines (Fang *et al.* 2005, 2007). The miRNA (miR)-29 family (29a, 29b and 29c) that has intriguing complementarities to the 3′-UTRs of DNA methyltransferase (DNMT)-3A and -3B (*de novo* methyltransferases) are downregulated in lung cancer. Inducing expression of miR-29s in lung cancer cell lines showed reactivation of methylation-silenced tumour suppressor genes such as *WWOX* and *FHIT*, and inhibited tumourigenicity *in vitro* and *in vivo* (Fabbri *et al.* 2007).

DNA hypomethylation can also promote carcinogenesis by activating oncogene transcription which is usually repressed in normal cells. Treating gastric cancer cells and colon cancer cells with S-adenosylmethionine (SAM), a methyl donor in biological transmethylation events inhibited cell growth by inducing promoter methylation of the oncogenes *c-Myc* and *H-ras*. However, there was no effect on the tumour suppressor gene *p16* (*INK4a*) with and without SAM treatment. Thus, SAM can be used to effectively downregulate oncogene expression and inhibit growth of tumour cells by reversing DNA hypomethylation on their promoters (Luo *et al.* 2010). In summary, these data indicate that reversing epigenetic patterns can induce expression or repression of a number of target genes that have a role in tumour progression. Clinical studies demonstrate that such agents, used alone or in combination, have proven to be effective in many cancer patients. Future research is essential to discover additional therapeutic targets regulating methylation pathways in cancer.

Conclusion

The Notch signalling pathway is involved in cell–cell communication and plays a critical role in cell fate determination. Deregulated Notch signalling is found to be involved in the development of various cancer types like cervical, ovarian, prostate, glioma, lung, pancreatic, etc. Notch genes can function as both oncogenes and tumour suppressor genes based on the type of tissue. Treating Notch signalling pathway appropriately using inhibitors or inducers can thus be a promising approach for cancer treatment.

Epigenetic mechanisms control gene expression patterns in developing and adult tissues. DNA methylation assures proper regulation of gene expression and stable gene silencing. When this genome protecting mechanism is disrupted by genotoxic agents, mutations occur as secondary events leading to cancer development. Both hypomethylation and hypermethylation are observed in cancer. Hypermethylated promoters of silenced genes are being used as biomarkers for diagnosis of cancer. Research on drugs that reverse DNA methylation are being widely carried out in cancer therapy and are found to be very effective *in vitro* and *in vivo*.

Methylation status of Notch pathway genes is studied in very few cancers. Much research on this topic needs to be done to draw conclusions on the role of methylated Notch genes in cancer development and their use as biomarkers in diagnosis and treatment.

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