DNA methylation regulates gene expression in normal cells. This epigenetic mechanism acts in at least two different ways: at global genomic level by targeting repetitive sequences distributed among the whole genome (LINEs, SINEs, satellite DNA, transposons) and at local level by targeting CpG islands in promoter regions. Both epigenetic mechanisms are involved in the carcinogenetic process; however, different evidences suggest that promoter hypermethylation occurring in genes involved in cell-cycle regulation, DNA repair, cell signalling, transcription and apoptosis likely plays a prominent role.

Opposite to genetic defects DNA hypermethylation is a reversible process that can be handled through “epigenetic drugs” in a wide spectrum of tumors. Along this line, recent data have demonstrated the ability of DNA hypomethylating agents to up-regulate and/or induce the expression of genes silenced by promoter hypermethylation in cancer. Particularly relevant seems the ability of these drugs to modulate the expression of genes coding for molecules crucial for tumor immunogenicity and immune recognition of neoplastic cells by host’s immune system, such as Cancer Testis Antigens, HLA class I molecules, costimulatory molecules. These evidences, coupled to the well-known cytotoxic, pro-apoptotic, and differentiating activities of epigenetic drugs, encourage to design and to develop new therapeutic strategies able to circumvent the immune escape of neoplastic cells and to potentiate the efficacy of immunotherapy in cancer patients.

This review will provide an update on the most recent information about aberrant DNA methylation in cancer and on innovative therapeutic strategies of “epigenetic remodelling” of human malignancies, with particular attention to the immunologic and immunotherapeutic potential of this approach.

**Summary.** DNA methylation regulates gene expression in normal cells. This epigenetic mechanism acts in at least two different ways: at global genomic level by targeting repetitive sequences distributed among the whole genome (LINEs, SINEs, satellite DNA, transposons) and at local level by targeting CpG islands in promoter regions. Both epigenetic mechanisms are involved in the carcinogenetic process; however, different evidences suggest that promoter hypermethylation occurring in genes involved in cell-cycle regulation, DNA repair, cell signalling, transcription and apoptosis likely plays a prominent role.

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cancer, in which the epigenetic “mark” is not properly established and/or maintained. In fact, global hypomethylation of the genome, leading to chromosomal instability, as well as hypermethylation of the promoter of genes involved in the control of proliferation, apoptosis and DNA repair, have been largely described in the initial phases of carcinogenesis (Feinberg and Vogelstein, 1983), indicating an increasing interest in the development of pharmacological agents able to revert these epigenetic abnormalities.

This review aims to present the most recent evidence on the role of DNA methylation in cancer, with particular attention to its involvement in the regulation of molecules that dictate the extent and the efficacy of the immune response against tumors. Moreover, based on recent evidence that has explored the ability of DNA hypomethylating agents (DHA) and/or of inhibitors of histone deacetylases to reactivate several genes aberrantly silenced in cancer, the most recent advances in the development of innovative therapeutic strategies based on the epigenetic remodelling of cancer cells will be reported.

Finally, the potential of DHA as immunological modulators in developing innovative, chemo-immunotherapeutic approaches in cancer patients will be discussed.

DNA methylation

The DNA methylation machinery

The mammalian DNA methylation machinery is composed of two main component blocks, the DNA methyltransferases (DNMTs) that establish and maintain DNA methylation patterns, and the methyl-CpG binding proteins (MBDs) that “read” methylation marks and dictate the levels of gene expression. In normal cells, DNA methylation occurs by covalent addition of a methyl group at the 5' carbon of the cytosine ring, resulting in 5'-methylcytosine (Bird, 2002).

These methyl groups bulge into the major groove of DNA and efficiently inhibit gene transcription. In mammalian DNA, 5'-methylcytosine is found in approximately 4% of genomic DNA, primarily at CpGs. These sites, the “CpG islands”, are typically found in or near gene promoter regions, where transcription is initiated.

Global genomic methylation

DNA methylation helps to maintain transcriptional silence of non-expressed and non-coding regions of the genome that reside in specific chromatin domains as pericentromeric heterochromatin, which is heavily methylated, tightly packaged, and transcriptionally inactive.

Global DNA methylation also occurs within repetitive elements that comprise about 45% of the human genome. These repetitive elements consist of interspersed repeats (LINEs, SINEs) and tandem repeats of simple sequences (satellite DNA) or of more complex sequences, whose significance and functions are still not fully understood. While LINEs and SINEs are interspersed throughout the genome, satellite DNA is largely confined to the centromeres or centromere-adjacent (juxtacentromeric) heterochromatin (Thornburg et al., 2006).

Global genomic methylation in normal cells ensures that these repetitive elements of genomic DNA remain late-replicating and transcriptionally quiescent, and suppresses the expression of any potentially harmful retroviral or transposon sequence that may have been integrated into sites containing highly repetitive sequences (SINEs, LINEs) (Fig. 1). On the contrary, hypomethylation of genomic DNA in cancer cells may favour mitotic recombination, leading to loss of heterozigosity and promotion of karyotypically detectable rearrangements (e.g., LINE-1 retrotransposons, and HERV-K proviral DNA) (Florl et al., 1999).

Local (Promoter) methylation

DNA methylation occurring at the promoter region of genes, represses transcription by inhibiting the binding of specific transcription factors and by recruiting MBDs and their associated repressive chromatin remodelling activities.

The distribution in the genome of the CpG dinucleotides at which DNA methylation occurs is asymmetric (i.e., occurs only at one of the two DNA strands). In contrast to the relative paucity of CpGs in the genome as a whole, these dinucleotides can be

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**Fig. 1. DNA methylation and cancer.**

A. Normal cells show a global genomic hypermethylation of non-coding DNA sequences (i.e. transposons, LINEs, SINEs) and a local gene promoter hypomethylation (occurring tumor suppressor genes, cell cycle regulator genes). B. In cancer cells, heterochromatin becomes hypomethylated and this provides an increased genomic instability. Moreover, CpG islands in promoter regions become hypermethylated leading to the transcriptional silencing of the gene. Black: 5'-methylcytosine White: normal cytosine (unmethylated).
clustered in small stretches of DNA, termed CpG islands, which are mostly associated with promoter regions (Feltus et al., 2003). It has been estimated that about 70% of genes contain at least one CpG island-containing promoter (Cheong et al., 2006).

Aberrant DNA hypermethylation contributes to tumorigenesis by enhancing DNA mutation rates (methyl-CpG are mutational hotspots) and, more importantly, by silencing specific genes. Interestingly, it has been reported that different sets of genes are silenced by aberrant DNA hypermethylation in different types of cancers, configuring the existence of tumor type-specific methylation patterns (Costello et al., 2000).

A wide spectrum of genes involved in the control of the cell cycle, DNA damage response, apoptosis pathway, cell invasiveness, all critical steps of the processes of cancer initiation and progression, are silenced by promoter hypermethylation (Fig. 1). For instance, Death-Associated Protein Kinase (DAPK), a pro-apoptotic serine/threonine kinase, is widely expressed in normal tissues but epigenetically silenced by promoter hypermethylation in various types of cancer such as B-cell lymphomas, non-small cell lung cancer, head and neck cancer, thyroid lymphoma, advanced stage gastric cancer and adenocarcinomas of the upper gastrointestinal tract (Wethkamp et al., 2006). Similarly, MLH1, a mismatch repair gene, is silenced by promoter hypermethylation in a subset of colon cancer, leading to microsatellite instability (Gras et al., 2001). Again, von Hippel-Lindau (VHL), a tumor-suppressor gene whose loss promotes tumor invasiveness and metastatic potential, is inactivated by promoter methylation in a subset of clear-cell renal carcinomas (Banks et al., 2006).

Recent studies have shown that abnormal epigenetic silencing occurs most frequently during the early stages of the neoplastic process (Baylin and Ohm, 2006), and involves most of the crucial cellular pathways. In particular, abnormal p16INK4a expression, silenced by methylation, was found in pre-invasive lung and breast tumors (Wong et al., 1999). Similarly, silencing of CDKN2A gene allows mammary epithelial cells to escape senescence, resulting in genetic instability (Ye et al., 2006). By targeting these genes, promoter hypermethylation provides tumor cells with a growth advantage, increases their genetic instability allowing them to acquire further advantageous genetic changes, and contributes to their metastatization.

A list of the most important tumor suppressor genes shown to be silenced or down-regulated in cancer by promoter hypermethylation, and which are involved in crucial cellular pathways is reported in Table 1.

**Regulation of the expression of immune molecules by methylation in cancer cells**

Besides regulating genes involved in functions critical for the cell, DNA methylation is also involved in regulating the expression of molecules crucial for the immune recognition of cancer cells by the host’s immune system.

In particular, a group of tumor-associated antigens (TAA), the Cancer Testis Antigens (CTA), has been extensively reported to be regulated by promoter methylation (De Smet et al., 1996; Sigalotti et al., 2002a). Likewise, the expression of molecules involved in antigen presentation in cancer cells has been reported to be controlled by methylation (Garcia-Lora et al., 2003; Sigalotti et al., 2005). Immune molecules known to be regulated by methylation are reported in Table 2.

**Cancer testis antigens**

CTA belong to a major family of TAA which share common features. In particular, CTA are clustered in multigene subfamilies largely mapping to the X chromosome, are immunogenic, and are not present in normal adult tissues (except for testis, fetal ovary and placenta), whereas they are expressed by tumor cells in a wide spectrum of human cancers, thus representing ideal targets for vaccine-based immunotherapy (van der Bruggen et al., 1994; Coral et al., 2002). However, observations which have emerged in the last years indicate that some obstacles could hamper this latter possibility. In particular, CTA: a) frequently show an heterogenous intra-tumor pattern of expression at molecular (Sigalotti et al., 2004) and protein level in human tumors (Roeder et al., 2005), b) therapeutic target CTA can be expressed in a limited percentage of patients.
(Akcakanat et al., 2006), and c) the amount of CTA expressed by neoplastic cells can be low, thus impairing their immune presentation (Sigalotti et al., 2004).

The prevalence of the expression of each individual CTA is variable between different tumor types. In particular, melanoma generally expresses several CTA from different subfamilies, as reported for sarcoma, lung cancer, breast cancer and prostate cancer. On the other hand, colon cancer, renal cancer and hematopoietic malignancies usually express a lower number of CTA. Interestingly, CTA tend to be expressed in “clusters” in tumors of the same histotype (Scanlan et al., 2002). In this respect, the analysis of the expression of a panel of seven CTA in 53 metastatic melanoma lesions demonstrated any of the evaluated CTA in about one tenth of specimens, whereas in 45% of cases at least 3 CTA were concomitantly expressed (Table 2).

The frequency of CTA expression in different neoplasias is highly variable. Recently, utilizing a large panel of epithelial ovarian cancer (EOC) specimens, Odunsi (2003) demonstrated that at least one of the two CTA under investigation (NY-ESO-1 and LAGE-1) was expressed in about 40% of cases (Odunsi et al., 2003). Along this line, Li (2005) studied the expression frequency of 10 selected CTA in 121 colorectal cancer (CRC) patients. The results showed that more than 56% of these CRC patients expressed at least one CTA. In particular, among them, about 27% of the specimens expressed more than two CTA (Li et al., 2005). Similarly, the expression of 12 selected CTA in 46 primary lung cancers was investigated in Japanese patients, showing that 20% of patients expressed one CTA, and the expression of 3 or more CTA was detected in 39% of patients (Tajima et al., 2003).

Globally, these observations prompted the development of polyvalent CTA vaccines that could widen the number of patients biologically eligible for CTA-directed immunotherapy and avoid immune-escape. This evidence describing CTA expression frequencies in tumor of different origin and histotype are summarized in Fig. 3 (Sahin et al., 1998, 2000; Odunsi et al., 2003; Tajima et al., 2003; Ayyoub et al., 2004; Li et al., 2005a; Akcakanat et al., 2006; Wu et al., 2006).

CTA expression has also been described to change along tumor progression: in one study, NY-ESO-1 expression has been found to be differentially expressed in colorectal cancer at different clinical stages, with higher frequencies in the advanced stages of disease (Bolli et al., 2005). Along this line, Goydos (2001) reported a similar behaviour also in melanoma, with NY-ESO-1 mRNA detected in 10% primary melanomas and in 47% of metastatic melanomas, thus suggesting that NY-ESO-1 can be a marker of advanced disease (Goydos et al., 2001).

### Table 1. Genes that are hypermethylated in different human cancers.

<table>
<thead>
<tr>
<th>Pathway or function</th>
<th>Hypermethylated genes</th>
<th>Biological effects</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wnt signalling</td>
<td>SFRP1, SFRP2, SFRP4,</td>
<td>Pathway Wnt activation, cell survival</td>
<td>Suzuki et al., 2004; Hiltunen et al., 1997</td>
</tr>
<tr>
<td></td>
<td>SFRP5, APC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mismatch repair</td>
<td>MLH-1</td>
<td>Defects in DNA mismatch-repair</td>
<td>Fukushima et al., 2005</td>
</tr>
<tr>
<td>DNA damage repair</td>
<td>BRCA1</td>
<td>Defects in DNA repair of double-strand breaks</td>
<td>Esteller et al., 2000</td>
</tr>
<tr>
<td>DNA damage repair</td>
<td>MGMT</td>
<td>Defective repair of guanosine methyl adducts</td>
<td>Baumann et al., 2006</td>
</tr>
<tr>
<td>P53-mediated DNA damage response</td>
<td>HIC1</td>
<td>Loss of apoptosis response to DNA damage</td>
<td>Fuji et al., 1998</td>
</tr>
<tr>
<td>P53 and Rb pathway</td>
<td>P14ARF</td>
<td>Activation of MDM2, inactivation of p53</td>
<td>Hsu et al., 2004</td>
</tr>
<tr>
<td>Detoxification and metabolism of xenobiotics</td>
<td>GSTP1</td>
<td>Deficient detoxification, accumulation of DNA adducts</td>
<td>Tew and Ronai, 1999</td>
</tr>
<tr>
<td>Tumor suppressor gene</td>
<td>VHL</td>
<td>Inactivation of tumor suppressor function</td>
<td>Peruzzi et al 2006</td>
</tr>
<tr>
<td>Cell cycle pathway</td>
<td>p16 (CDKN2A), p15 (CDKN2B)</td>
<td>Costitutive activation of cell cycle</td>
<td>Attiri et al., 2005, Aggerholm et al., 2006</td>
</tr>
<tr>
<td>Transcription factors</td>
<td>GATA4, GATA5</td>
<td>Defective epithelial cell differentiation</td>
<td>Gao et al., 1998</td>
</tr>
<tr>
<td>Cell invasion</td>
<td>TIMP3</td>
<td>Inhibition of tissue metalloproteinases</td>
<td>Bachman et al., 1999</td>
</tr>
<tr>
<td>Cell-cell adhesion</td>
<td>CDH1 (E-cadherin)</td>
<td>Defective cell invasiveness</td>
<td>Yoshiura et al., 1995</td>
</tr>
<tr>
<td>Apoptosis pathway</td>
<td>APAF-1</td>
<td>Defective intrinsic pathway of apoptosis</td>
<td>Furukawa et al., 2005</td>
</tr>
<tr>
<td>Apoptosis pathway</td>
<td>DAPK</td>
<td>Inhibition of apoptosis</td>
<td>Reddy et al., 2003</td>
</tr>
<tr>
<td>Apoptosis pathway, cell cycle pathway</td>
<td>RASSF1</td>
<td>Inhibition of apoptosis, lack of growth control</td>
<td>Dammann et al., 2005</td>
</tr>
<tr>
<td>Receptor of Retinoic acid</td>
<td>RARß2</td>
<td>Lack of response to retinoids</td>
<td>Li et al., 2005b</td>
</tr>
<tr>
<td>Cellular binding retinol protein</td>
<td>CRBP1</td>
<td>Lack of response to retinoids</td>
<td>Esteller et al., 2002</td>
</tr>
<tr>
<td>Estrogen receptor-α</td>
<td>ER-α</td>
<td>Defective growth of breast cells</td>
<td>Fuji et al., 2005</td>
</tr>
</tbody>
</table>
Except for selected CTA (i.e., SCP-1, OY-TES-1), the biological function of most of them is largely unknown; in particular SCP-1 gene, encoding for the synaptonemal complex protein 1, is involved in the pairing of homologous chromosomes (Tureci et al., 1998), whereas OY-TES1 gene encodes for SP32 precursor, a protein that binds to proacrosin and is involved in the packaging and condensation of the acrosin zymogen in the acrosomal matrix (Ono et al., 2001).

Recent studies have revealed that MAGE family proteins could have roles in cell cycle progression and apoptosis, because they have been shown to interact with key molecules for cell cycle and apoptosis like p53, E2F1 (Kuwako et al., 2004; Monte et al., 2006) as well as with the neurotrophic factor, p75NTR, which is expressed by neural-crest-derived stem cells (Takaki et al., 2006). However, further studies are needed to fully elucidate their precise functions.

Several reports have investigated the mechanisms regulating CTA expression, and it is well-established that the regulation of the expression of CTA is determined by promoter methylation status (De Smet et al., 2004) and/or by other epigenetic modifications operated by histone acetyltrasferases and histone deacetylases (Wischnewski et al., 2006). In particular, our previous studies identified promoter methylation as a mechanism directly regulating the expression of CTA in metastatic melanomas (Sigalotti et al., 2002a). Moreover, utilizing single cell clones obtained from a primary cell culture generated from a human cutaneous metastatic melanoma lesion, we recently demonstrated that a differential promoter methylation is directly responsible for the intratumoral heterogeneity of therapeutic CTA in melanoma (Sigalotti et al., 2004). These observations suggested the use of 5-aza-2'-deoxycytidine (5-AZA-CdR, decitabine) to overcome the limitations set by their heterogeneous intratumor expression to CTA-based vaccine therapy. In fact, it has been suggested that 5-AZA-CdR could revert the CTA-

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Table 2. Genes coding for immune molecules that are methylation-regulated.

<table>
<thead>
<tr>
<th>Methylation-regulated immune molecules</th>
<th>Biological functions</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>MAGE-A family</td>
<td>p53 repressor</td>
<td>Monte et al., 2006</td>
</tr>
<tr>
<td>SSX family</td>
<td>Transcriptional repressor</td>
<td>de Bruijn et al., 2002</td>
</tr>
<tr>
<td>SCP-1</td>
<td>Protein involved in pairing of homologous chromosomes</td>
<td>Tureci et al., 1998</td>
</tr>
<tr>
<td>OY-TES-1</td>
<td>Binding protein involved in packaging and condensation of the acrosin zymogen in the acrosomal matrix</td>
<td>Ono et al., 2001</td>
</tr>
<tr>
<td>NY-ESO-1, LAGE-1</td>
<td>Unknown</td>
<td>Chen et al., 1997</td>
</tr>
<tr>
<td>HLA class I antigens</td>
<td>Antigenic peptide presentation</td>
<td>Fonsatti et al., 2003</td>
</tr>
<tr>
<td>CIITA</td>
<td>Transactivators of HLA class II antigens</td>
<td>Morris et al., 2000</td>
</tr>
<tr>
<td>I-CAM1/CD54</td>
<td>Cytokine involved in immune response</td>
<td>Hellebrekers et al., 2006</td>
</tr>
<tr>
<td>CD59</td>
<td>Complement-regulatory proteins</td>
<td>Kuraya et al., 1993</td>
</tr>
</tbody>
</table>

Fig. 3. Expression frequencies of CTA in tumors of different histotype. EOC: Epithelial Ovarian Cancer; CRC: Colorectal Cancer; MM: malignant melanoma; HCC: Hepatocarcinoma; White background, black dots: not present; Grey background, white dots: rare; Black background, white dots: ≥ 1
negative or weakly-positive phenotype of distinct melanoma cells within neoplastic lesions, generating a population of neoplastic cells homogeneously expressing the therapeutic target CTA (Sigalotti et al., 2004).

Along this line, increasing evidence in vitro showed that treatment with 5-AZA-CdR is able to enhance or to induce a de novo expression of CTA in tumors of different origins, such as sarcoma (Ayyoub et al., 2004), colon cancer (Fang et al., 2003), mesothelioma (Sigalotti et al., 2002b), as well as in different hematological malignancies (Sigalotti et al., 2003; Calabrò et al., 2005).

These data are intriguing as they suggest that treatment with DHA could increase the therapeutic efficacy of active and/or adoptive CTA-based immunotherapeutic strategies in different human malignancies.

Antigen presentation

HLA class I and class II molecules play essential roles in the immune response by virtue of their function in presenting antigenic peptides to T lymphocytes. Given their central role in antigen-specific immune response, the genes encoding for these molecules are regulated in a tight fashion to meet the requirements for an adequate immune response. In fact, presentation of antigenic peptides in the context of HLA molecules is crucial both during T cell priming and during the effector phase of an adaptive immune response.

Nearly all human cells express HLA class I molecules, whereas HLA class II are physiologically expressed only by immune cells; however, several tissues are characterized by a significantly reduced membrane expression of HLA class I molecules. In particular, the expression of HLA class I molecules is lacking in reproductive and developmental tissues (sperm, oocytes, preimplantation embryos, and villous trophoblast cells) which intriguingly express CTA.

Although alterations of HLA class I and class II antigens expression have been extensively reported to be due to gene mutations or deletions (Browning and Dunnion, 1997), emerging evidence points to a fundamental role for an epigenetic regulation of many genes encoding for components of the antigen presentation machinery (Garcia-Lora et al., 2003; Sigalotti et al., 2005). In fact, promoter hypermethylation of HLA class I genes was shown to correlate with the loss of expression of HLA class I in human esophageal squamous cell carcinomas (Nie et al., 2001). As far as melanoma is concerned, our previous findings demonstrated that the constitutive levels of expression of HLA class I antigens were significantly up-regulated by the exposure to 5-AZA-CdR (Coral et al., 1999). Along this line, the immunobiologic properties of DHA in cancer were also highlighted by the observations that 5-AZA-CdR treatment concomitantly up-regulated the expression of other components of the tumor recognition complex (i.e., Interacellular Adhesion Molecule-1, Leukocyte-Function Associated Antigen-3) (Coral et al., 1999; Calabrò et al. 2005).

Similarly, recent data showed that the lack of MHC class II expression on leukemic T cells is caused by hypermethylation of their transactivators CIITA, resulting in the lack of MHC class II expression (Holling et al., 2004). Based on this evidence, a central role for DNA methylation in the control of the expression of HLA class I and HLA class II genes in various tumor cell types has emerged, representing an additional mechanism operated by tumor cells to escape immune surveillance (Wright and Ting, 2006).

DNA methylation as a therapeutic target in cancer

As shown by the evidence reported so far, the pharmacological modulation of the epigenetic profile of neoplastic cells, such as histone acetylation/deacetylation, histone methylation and, in particular, DNA methylation, may allow the development of innovative approaches for the therapy of human cancer.

These epigenetic changes lead to the loss of function of genes similar to that induced by gene mutations. However, genetic and epigenetic defects display fundamental differences, which carry profound clinical implications. In particular, whereas genetic mutations cannot be reverted, epigenetic gene silencing can be reverted by pharmacological agents. Among currently available DHA, able to revert these epigenetic modifications in a non-specific way, 5-aza-cytidine (azacytidine) and 5-AZA-CdR have been demonstrated to be suitable agents for “epigenetic therapy”.

DHA are incorporated into DNA, resulting in the inhibition of DNMTs activity through covalent binding, leading to global hypomethylation in most human cancer cell lines tested (Esteller, 2003). Moreover, other compounds such as non-nucleoside inhibitors and antisense oligodeoxynucleotides, which directly inhibit DNMTs and do not need to be incorporated in DNA have been developed (Hellebrekers et al., 2007).

Treatment of tumor cells with 5-AZA-CdR is able to reactivate their expression of genes involved in the control of apoptosis, proliferation, DNA repair and cellular senescence, which are crucial to the development of cancer (Neumeister et al., 2002). Additionally, these drugs also induce a series of phenotypic changes that might potentiate their antitumor effect. As previously summarized, 5-AZA-CdR, besides modulating CTA expression, also positively affects the expression of selected components of the “tumor recognition complex” (HLA class I antigens and allospecificities, co-stimulatory molecules). These modifications, altogether, enhance the recognition of cancer cells by cytotoxic T cells, thus improving the targeting of T-lymphocytes to the tumor (Sigalotti et al., 2004). In this respect, consistent with data obtained with human melanoma xenografts in BALB/c nu/nu mice (Coral et al., 2006), Guo (2006) recently demonstrated...
that combining adoptive immunotherapy with CTLs, raised against the murine CTA P1A, and treatment with DHA enhances the efficacy of immune T recognition of syngeneic mammary cancer cells in vivo (Guo et al., 2006).

As far as melanoma is concerned, these observations suggest that 5-AZA-CdR could widen the biological eligibility of CTA-directed immunotherapy to virtually all patients, as also encouraged by the long-lasting effects of DNA hypomethylating agents in modulating the antigenic profile of tumor cells in vitro and in vivo (Coral et al., 2007).

Advances in the design of specific and potent drugs to prevent or overcome the effects of DNA methylation require, however, more information on the mechanisms by which gene silencing is established.

Clinical studies on DNA hypomethylating agents

In human cancer, exposure to 5-AZA-CdR has been reported to have dual effects on treated cells, depending on the dose of drug administrated. At high doses, 5-AZA-CdR induces apoptotic cell death triggered by the production of DNA adducts and consequent DNA synthesis arrest (Juttermann et al., 1994), whereas at low doses it leads to reduced proliferation, cellular senescence and/or increased apoptosis (Jones and Taylor, 1980).

These compounds and their derivatives have been introduced into the clinic with encouraging results in haematopoietic disorders such as myelodisplastic syndrome (MDS) and acute myeloid leukaemia (AML) (Wijermans et al., 2000).

In a phase I clinical study, Issa (2004) demonstrated that low doses of decitabine are clinically effective in AML, MDS, and chronic myelogenous leukemia (CML) (Issa et al., 2004). Moreover, in patients affected by MDS, AML or CLL, treatment with decitabine was shown to induce CTA expression in transformed cells of hematopoietic origin, thus providing evidence that it can “rescue” CTA as immunological targets (Sigalotti et al., 2003). Available data about clinical use of DHA have led to the registration of 5-azacitidine (VIDAZA) and decitabine (DACOGEN) by the Food Drug Administration in the United States and by European Medicines Agency (EMEA) for the treatment of high risk MDS.

Recent clinical evidence has also shown that low doses of decitabine induce objective responses in solid tumors. A recent phase I study was designed to evaluate the feasibility and the toxicity of prolonged 5-AZA-CdR infusion of decitabine in patients with lung and esophageal cancers, as well as malignant pleural mesothelioma. This study also demonstrated that treatment with decitabine induces or up-regulates the expression of CTA, such as other aberrantly methylation-silenced genes (e.g., p16) also in this setting (Schrump et al., 2006).

Along this line, recent results of a phase I clinical study of the combined treatment with decitabine plus high-dose interleukin-2 (IL-2) in melanoma and renal cell carcinoma suggest that DHA may synergistically act with immunological treatments also in the clinical setting (Gollob et al., 2006).

Despite the promising data arising from clinical trials, there are several pitfalls regarding the clinical application of demethylating agents. The inherent toxicity of nucleoside DNMT inhibitors might be caused by the formation of covalent adducts between DNA and trapped DNMTs. Furthermore, the majority of the demethylating drugs available are not specific for a particular DNMT or gene, which can result in widespread DNA hypomethylation and the production of unfavourable effects. Toxicity, a central problem in the clinical utilization of these drugs, might be decreased by optimising treatment schedules, e.g., giving lower doses over longer time periods, thereby exposing more cells to the drug during the S phase. Also, the development of novel non-nucleoside inhibitors of DNMTs (e.g., hydralazine, procainamide) could lead to lower toxicity, as these compounds are not incorporated into DNA (Chuang et al., 2005). Moreover, DHA that specifically target a particular DNMT, such as MG98, an antisense oligonucleotide targeting the mRNA of the enzyme DNMT1, might also reduce these nonspecific effects (Lee et al., 2005).

Another important point that should be taken into account in the clinical use of DHA is the widespread induction of promoter hypomethylation, which might induce tumorigenesis by activation of oncogenes (Gaudet et al., 2003). In this respect, the global and the gene-specific DNA methylation changes were recently examined in leukaemia patients treated with decitabine. The results suggested, according to several in vitro studies, that although a dose-dependent global hypomethylation can be observed, genes aberrantly imprinted by promoter hypermethylation seem particularly sensitive also to low doses of the epigenetic drugs in cancer cells (Yang et al., 2006).

Combined epigenetic therapies

Increasing evidence indicate that in the development of drug-resistance of cancer cells, epigenetic changes are likely to be a driving force. Along this line, several studies have shown that epigenetic drugs are able to revert drug resistance or to increase the cytotoxicity of anticancer drugs and radiation (Plumb et al., 2000; Kim et al., 2003; Qiu et al., 2005; Sonneman et al., 2006). In a recent study, the effect of hydralazine, a non-nucleoside demethylating agent, combined to valproic acid, a histone deacetylase inhibitor, was evaluated in a panel of cell lines from different tumor types. The results of this study showed that hydralazine and valproic acid have promising antineoplastic effects and have the ability to up-regulate a number of genes, both in vitro and in vivo; in addition, this combined treatment seemed to increase the antitumor efficacy of current cytotoxic
agents (gemcitabine, cisplatin, adriamicin) (Chavez-Blanco et al., 2006).

Preclinical information from experimental models using DNA methylation and histone deacetylase inhibitors suggest that epigenetic therapy could erase the epigenetic marks associated with the chemotherapeutic-resistant phenotype and, therefore, sensitize tumors to chemotherapy (Perez-Plasencia and Duenas-Gonzalez, 2006). On this point, the results of a recent study by Segura-Pacheco (2006) in the MCF-7/Adr model, demonstrated that promoter DNA hypermethylation participates in the development of adriamycin resistance and that hydralazine, another demethylating agent, can revert the resistant phenotype (Segura-Pacheco et al., 2006). Moreover, the same group is planning a phase II study that will evaluate the effects of hydralazine and magnesium valproate, an histone deacetylase inhibitor, in resensitizing tumor cells to chemotherapy in refractory solid tumors, as well as their ability to improve tumor response to adriamycin-based neoadjuvant therapy in locally advanced breast cancer.

The multidrug resistance represents one of the major hurdles to effective chemotherapy also in neuroblastoma. Along this line, recent evidence demonstrated that treatment of transfected murine neuroblastoma cells with 5-AZA-CdR at non-toxic doses, sensitized xenografts to cisplatin, carboplatin, temozolomide and epirubicin (Qiu et al., 2005).

Overall, these observations support the potential role of epigenetic therapy in reverting the chemotherapy-resistant phenotype of malignant tumors, and in increasing the efficacy of chemotherapeutic drugs.

Conclusions

On the basis of the evidence described above, the fundamental role of DNA methylation in human cancer initiation and progression is clearly emerging. In particular, its involvement in the control of the expression of genes engaged in cell-cycle regulation, tumor cell invasion, DNA repair, cell signalling and apoptosis, as well as of genes encoding for “immune molecule” like CTA, HLA class-I and HLA class-II antigens, strongly promotes the development of new therapeutic approaches based on “epigenetics drugs”. Preclinical and clinical data currently available strongly indicate that “demethylating therapy” can efficiently revert the aberrant DNA methylation patterns associated with cancer.

The regulation of genes by epigenetic drugs involved in cell cycle and apoptosis control likely enhances the susceptibility of tumor cells to apoptotic cell death, which could potentiate the effects of current anticancer therapies (i.e. chemotherapy, radiotherapy, immunotherapy).

In addition, the potentiating activity of DHA could be even more effective for immunological therapies, through their effects in enhancing immunogenicity and immune recognition of tumor cells, strongly configurating the need for the development of new combined chemio-immunotherapeutic approaches in human malignancies.

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