Epigenetic mechanisms in hepatocellular carcinoma: How environmental factors influence the epigenome

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ARTICLE INFO

Article history:
Received 5 January 2011
Received in revised form 5 April 2011
Accepted 6 April 2011
Available online 15 April 2011

Keywords:
Epigenetics
Cancer risk-factor
Environment
DNA methylation
Histone modifications
Noncoding RNAs
Liver cancer

ABSTRACT

Epigenetic mechanisms maintain heritable changes in gene expression and chromatin organization over many cell generations. Importantly, deregulated epigenetic mechanisms play a key role in a wide range of human malignancies, including liver cancer. Hepatocellular carcinoma (HCC), which originates from the hepatocytes, is by far the most common liver cancer, with rates and aetiology that show considerable geographic variation. Various environmental agents and lifestyles known to be risk factors for HCC (such as infection by hepatitis B virus (HBV) and hepatitis C virus (HCV), chronic alcohol intake, and aflatoxins) are suspected to promote its development by eliciting epigenetic changes, however the precise gene targets and underlying mechanisms have not been elucidated. Many recent studies have exploited conceptual and technological advances in epigenetics and epigenomics to investigate the role of epigenetic events induced by environmental factors in HCC tumors and non-tumor precancerous (cirrhotic) lesions. These studies have identified a large number of genes and pathways that are targeted by epigenetic deregulation (changes in DNA methylation, histone modifications and RNA-mediated gene silencing) during the development and progression of HCC. Frequent identification of aberrant epigenetic changes in specific genes in cirrhotic tissue is consistent with the notion that epigenetic deregulation of selected genes in pre-malignant lesions precedes and promotes the development of HCC. In addition, several lines of evidence argue that some environmental factors (such as HBV virus) may abrogate cellular defense systems, induce silencing of host genes and promote HCC development via an “epigenetic strategy”. Finally, profiling studies reveal that HCC tumors and pre-cancerous lesions may exhibit epigenetic signatures associated with specific risk factors and tumor progression stage. Together, recent evidence underscores the importance of aberrant epigenetic events induced by environmental factors in liver cancer and highlights potential targets for biomarker discovery and future preventive and therapeutic strategies.

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1. Introduction

Hepatocellular carcinoma (HCC) is a well-recognized complication of cirrhosis. Infection by hepatitis B (HBV) and hepatitis C HCV viruses, chronic alcoholism, aflatoxin exposure, smoking and
and synergistic interactions between several abnormal epigenetic and genetic events. The susceptibility of hepatocytes to neoplastic transformation is further influenced by their epigenetic and genetic state. The onset and progression of HCC is often caused by mutual damage rates and high metabolic rates, all of which increase the possibility of disruption of both genetic and epigenetic events in hepatocytes. In cirrhotic liver tissue, inflammatory responses and cirrhosis. Inflammatory and immune responses are accompanied by cytokine production, generation of reactive oxygen species, increased DNA damage rates and high metabolic rates, all of which increase the possibility of disruption of both genetic and epigenetic events in hepatocytes.

Fig. 1. Genetic and epigenetic events in the onset and progression of hepatocellular carcinoma. Infections with hepatitis B and C viruses, alcoholism, exposure to aflatoxin, hypertension and diabetes are the major HCC associated risk factors. Through different mechanisms exposure to various risk factors induces hepatocellular injuries, inflammatory responses and cirrhosis. Inflammatory and immune responses are accompanied by cytokine production, generation of reactive oxygen species, increased DNA damage rates and high metabolic rates, all of which increase the possibility of disruption of both genetic and epigenetic events in hepatocytes. In cirrhotic liver tissue hepatocytes susceptibility to neoplastic transformation is further influenced by their epigenetic and genetic state. The onset and progression of HCC is often caused by mutual and synergistic interactions between several abnormal epigenetic and genetic events.

possibly obesity and diabetes are believed to be the major risk factors associated with the incidence of HCC (Fig. 1). Regardless of aetiology the risk of malignancy is determined by the underlying cause of liver damage, which is further influenced by age, gender and ethnic differences in environmental and lifestyle factors [1–3]. Both genetic and epigenetic factors form the molecular basis of HCC. Although sequences of HBV are found integrated in the genome of HCC cells and virus-related insertional mutagenesis occurs frequently in liver cancers, there is no consensus pattern of viral integration [4,5]. A frequent loss of heterozygosity (LOH) in chromosome 8p in HCC cases suggests that inactivation of the Deleted in Liver Cancer 1 gene (DLC-1) may play a pivotal role in HCC development [2]. In late stages of HCC development, somatic mutations in several tumor suppressor genes (such as TP53, p16, and RB), oncogenes (including c-MYC and β-catenin) and other cancer-associated genes (including E-cadherin and cyclin D1) have also been observed [2,6]. However, the significance and sequence of these genetic events remain to be established. While germline mutations are reflected in the familial cancer history, a risk factor-associated somatic mutation should be noticeable in most of the cases exhibiting HCC associated with a particular risk factor. In either case, such genetic events would have been quite evident and with relatively rapid consequences. Contrary to this, the onset and development of HCC is a lengthy (several years) process with distinct stages of progression. The large time gap between initial exposure to the risk factor(s) and HCC onset indicates that consistent risk factor exposure, cirrhosis and lifestyle and environmental cues may result in establishing an aberrant epigenetic stage for HCC onset (Fig. 1).

The term “epigenetics” refers to all stable changes of phenotypic traits that are not coded in the DNA sequence itself [7–10]. Epigenetic mechanisms can be viewed as an interface between the genome and risk factor/life style/environmental influence. Aberrant epigenetic events associated with any of these stressors likely play an important role in the onset and progression of different human malignancies. The field of epigenetics has been receiving remarkable attention recently, owing to our increased awareness that epigenetic inheritance is essential for the development of critical cellular processes such as gene transcription, differentiation and protection against viral genomes. Aberrant epigenetic states may predispose to genetic changes, but genetic changes may also initiate aberrant epigenetic events. Epigenetic and genetic mechanisms may thus work together to silence key cellular genes and destabilize the genome, leading to oncogenic transformation and observed the complexity and heterogeneity in human cancers, including HCC [2,3,8].

2. HCC and aberrant DNA methylation changes

DNA methylation is a major epigenetic mechanism of gene regulation occurring in eukaryote DNA at CpG sites, usually enriched in the promoters of genes. In a wide range of tumors, including HCC, global hypomethylation and specific promoter hypermethylation have been linked with genomic instability and inactivation of tumor suppressor genes (TSG), respectively [7–9,11,12].

Aberrant DNA methylation changes have been reported to be specific to the cancerous tissue making it possible to distinguish HCC and non-HCC surrounding liver tissues. Abnormal DNA methylation of RASSF1A, p16, CRABP1, GSTP1, CHRNA3, DOK1, SFRP1, GAAD45a and p15 tumor suppressor genes is associated with HCC, while hypermethylation of the CHFR (checkpoint with forkhead associated and ring finger) and SYK (spleen tyrosine kinase) is detected specifically in advanced stages of HCC. It is well established that DNA methylation changes unlike genetic events, occur in a gradual manner. Methylation of multiple CpG sites in a CpG island located in the promoter region of a gene leads to the complete silencing of gene-expression e.g. multiple methylations are required for silencing of tumor suppressor genes (p16, RASSF1, etc.) [7–9,13]. This further underlines the specificity of HCC associated epigenetic events and helps clarify the large time gap between the initial risk factor exposure and HCC onset. Moreover,
HCC tumors exhibit specific DNA methylation signatures associated with major risk factors and tumor progression stage, highlighting potential clinical applications of these epigenetic changes in the diagnosis and prognosis of HCC [13–17] (Table 1).

Interestingly, distinct methylation of several independent panels of gene promoters has also been strongly correlated with survival after cancer therapy. The exact molecular mechanism governing the risk factor-specific hypermethylation signature (epigenotype) is unclear and remains an area of much current research interest [13,18].

To better explain how a given risk factor may affect the hepatocyte epigenome, leading to the neoplastic transformation, we take the example of infection with HBV a well-known and widely studied HCC risk factor. The presence of HBV in host hepatocytes may result in both genetic (insertional mutation) and epigenetic events (Fig. 2). In HBV infection-associated HCC's, the HBV encoded protein X (HBx) was found to upregulate expression of the DNA-methyltransferase (DNMT) genes through its transcriptional transactivation property, thus the corresponding down-regulation of several important cellular genes has been attributed to the DNMT-mediated methylation of the target genes [6,13–20]. HBx may also directly interact with DNMT's, directing their recruitment at specific genes and thus affecting their methylation and expression. Methylation of some TSGs, such as the SOCS-1, GADD45b, STAT1, APC, and p15 genes, is reportedly observed at a higher prevalence in HCV-positive than in HCV-negative HCC.

Table 1

<table>
<thead>
<tr>
<th>HCC risk factor</th>
<th>Reported epigenetic event</th>
<th>Contribution in HCC development</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>HBV infection</td>
<td>DNA methylation</td>
<td>Alterations of RB1, p53 and Wnt pathways due to silencing of important tumor suppressor genes (p16, p21, E-cadherin and GSTP1)</td>
<td>[13–17]</td>
</tr>
<tr>
<td></td>
<td>Histone modifications</td>
<td>Altered expression of critical cellular genes (hTERT, IGFBP-3 interleukin-4 receptor and metallothionein-1F and CDH6)</td>
<td>[23–26]</td>
</tr>
<tr>
<td></td>
<td>RNA interference</td>
<td>mirl-152, mirl-602 and mirl-143 MIR regulate important cellular genes including DNMT1, RASSF1A and FNDC3B affecting pathways related to cell death, DNA damage, recombination, and signal transduction.</td>
<td>[15,27–30]</td>
</tr>
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<td></td>
<td>Histone modifications</td>
<td>Increased histone deacetylation activity regulates iron metabolism through affecting hepcidin expression. Overexpression of Protein Phosphatase 2A (PP2Ac), affecting the H4 acetylation and methylation and histone H2AX phosphorylation.</td>
<td>[9,21,32,33]</td>
</tr>
<tr>
<td></td>
<td>RNA interference</td>
<td>mirl-122 regulates HCV replication. mirl-196 regulates HMOX1/Bach1 and HCV expression.</td>
<td>[34,35]</td>
</tr>
<tr>
<td>Alcoholism</td>
<td>DNA methylation</td>
<td>Hypermethylation of MGMT affects the DNA repair efficiency. CYP2E1 down regulation results in decreased mitochondrial oxidative stress and apoptotic potential. Adh, GST-yc2 are upregulated, while Lsdh, cytp450s2c1 are downregulated</td>
<td>[9,21,32,33]</td>
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<td>Histone modifications</td>
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<td>[36,37]</td>
</tr>
<tr>
<td>Aflatoxin exposure</td>
<td>DNA methylation</td>
<td>p16 silencing and hypomethylation mediated overexpression of SNCG</td>
<td>[38,39]</td>
</tr>
<tr>
<td></td>
<td>RNA interference</td>
<td>p1622s expression and function is affected (skewed IGF-1R regulatory circuitry)</td>
<td>[40]</td>
</tr>
</tbody>
</table>

Fig. 2. A hypothetical model depicting potential genetic and epigenetic changes induced by HBV infection and integration of the viral genome that may promote hepatocarcinogenesis. (1) HBV genome tends to integrate within the host cell-genome, which may result in insertional mutations of critical cellular genes. However no consistent pattern of insertional mutation has been noticed among different HCC patients HCC liver samples [2,5,53]. (2) The HBV encoded protein X (HBx) directly interacts with different cellular nuclear proteins and affects cellular processes (such as DNA damage repair) resulting in high mutational rates due to a compromised DNA repair capacity and integrity of cellular genome [54,55]. It is hypothesized that being the DNA of a foreign pathogen, the HBV genome may be targeted for methylation-mediated silencing by host surveillance machinery and the gradual spread of DNA hypermethylation may affect nearby genes or enhancers. Alternatively, activation of the host surveillance mechanism may exhibit long-range effects resulting in hypo-/hyper-methylation of other parts of the host genome. (3 and 4) HBV genome exists as a minichromosome in the host cell and uses host cellular machinery for its transcription and replication, which is further regulated epigenetically by histone modifications and DNA methylation [19,44,48,56]. HBx may interact with and affect histone modification enzymes [24,26,47,57]. Hijacking/interfering with the host cellular transcriptional/epigenetic machinery may result in an aberrant epigenetic state of the host cell genome making it susceptible to neoplastic transformation.
tumors [3,4,21,22]. However, HCV itself, which is known as an RNA virus, does not integrate into and directly disturb the host genome. Interestingly, HCC epigenotype also depends on the age and gender of the patient and has more in common with the HBV and HCV virus-associated HCC, compared to that observed in alcohol, aflatoxin or other lifestyle-associated HCC.

In addition to altering promoter specific methylation patterns, HCC onset and progression is also associated with genomic instability accompanied by the activation of oncogenes or transposons, which in turn may promote tumorigenesis by altering methylation states of repetitive sequences. Cancer cell genomes undergo global hypomethylation, with malignant cells having 20–60% less genomic 5-methylcytosine than their normal counterparts [41]. This loss is accomplished mainly by hypomethylation of the repetitive DNA sequences that account for 20–30% of the human genome [41]. The methylation level of LINE1 repetitive element and satellite 2 (SAT2) in pericentromeric satellite regions decreases along with the progression of chronic hepatitis and HCC [6,7,11,38,39,42]. Expression of DNMT3B4, which may lack DNA methyltransferase activity and compete with DNMT3B3 in targeting pericentromeric satellite regions results in DNA hypermethylation on SAT2 and plays a critical role in inducing chromosomal instability [43]. DNA methylation is also used as a common cellular defense mechanism to silence invading foreign DNA and viral genomes. Several recent studies have investigated whether HBV DNA is targeted for methylation in the infected hepatocytes (Fig. 2). Moreover, as the double-stranded DNA genome of HBV contains distinct CpG islands in the promoter and enhancer regulatory region for the viral genes, it has been argued that hypermethylation may silence the HBV surface antigen gene resulting in occult HBV cases, wherein patients with HCC test negative for hepatitis B surface antigen (HBsAg) although their liver remains infected with HBV [42,44]. Investigations have revealed that the HBV genome is hypermethylated in both premalignant (cirrhosis) and malignant (HCC) tissues, compared with nontumor liver tissues, suggesting that hypermethylation of the viral genome must also be strongly associated with the malignant process. However, patterns of HBV genome methylation in cirrhotic and tumor tissues from both occult and non-occult HBV-infected samples reportedly remain similar. This indicates that hypermethylation of the HBV genome resulting from deregulated DNA methylation in malignant cells may contribute to the disease phenotype, but is unlikely to be responsible for the occult status. Therefore, the exact biological significance of the observed methylation in HBV genome and its role in HCC remains unclear [44,44].

3. Aberrant histone modification changes in HCC

Recent studies have revealed the importance of histone-modifying enzymes and ATP-dependent chromatin remodeling complexes in regulating access to DNA for various transcription factors and DNA repair proteins necessary for catalyzing the critical chromatin activities of transcription, replication or DNA repair. Histone (chromatin) modifications comprise covalent post-translational modifications of histone proteins. The N-terminal tails of nucleosomal histones are subject to different modifications, including acetylation, methylation, phosphorylation and ubiquitination, which appear to work together with other epigenetic mechanisms in establishing and maintaining gene activity states, thus regulating a wide range of cellular processes. Different histone modifications themselves act in a coordinated and orderly fashion to regulate cellular processes such as gene transcription, DNA replication and DNA repair. Alterations in the function of histone-modifying molecules and protein complexes disrupt the pattern and levels of histone marks and consequently deregulate the control of chromatin-based processes, which ultimately leads to oncogenic transformation and development of cancer [8,9,12,45].

HCC has been reported to display altered histone modification machinery and as a result an altered cellular epigenetic state. Most of the known HCC-associated aberrant histone modification events affect expression of critical cellular genes and thus impair normal cellular activities (Table 1). Several such modifications are associated with the onset of HCC and act as a susceptibility factor for HCC, e.g. Ptt1 (a GNAT family acetyltransferase) is highly expressed in a normal healthy liver and is downregulated in HCC. Low levels of this acetyltransferase result in a hypoacetylated (inactive) state of apoptotic genes, affecting the apoptotic potential of cancerous hepatocytes [23–26]. Similarly, dimethylation of histone H3 at lysine 4 (H3K4diMe) is expressed at almost undetectable levels in HCC. These low levels of the H3K4diMe histone mark, were found to be caused by compromised expression of H3K4 methylating (Ash2 complex) and demethylating enzymes (LSD1) [46].

Certain histone code alterations are a signature for specific risk factor exposures e.g. HCV infection induces an overexpression of Protein Phosphatase 2A (PP2Ac), which binds to protein arginine methyltransferase 1 (PRMT1) and inhibits its activity. PRMT1 catalyzes the methylation of histone H4 on arginine 3 and also plays an important role in DNA repair by dephosphorylating the damage-induced phosphorylation of H2AX (γ-H2AX). Hence, PP2Ac overexpression in HCV-associated HCCs leads to compromised histone H4 methylation/acetylation and histone H2AX phosphorylation, significantly changing the expression of genes important for hepatocarcinogenesis and inhibiting DNA damage repair. In fact, overexpression of this important phosphatase is considered a critical early event in hepatocarcinogenesis in the context of chronic viral hepatitis [9,21,32]. In alcohol-associated HCCs it is suggested that deregulated histone modification down-regulates CYP2E1 expression, resulting in decreased mitochondrial oxidative stress and apoptotic potential [36,37]. HBV-encoded HBx protein is considered an oncogenic transcription factor and has been proven to affect the expression of important cellular genes due to its transcription transactivation and transrepression properties. HBx has been reported to induce cellular transformation by affecting the expression of numerous genes involved in the control of the cell cycle or apoptosis. The transcription transactivation property of HBx is supported by the fact that HBx directly interacts with histone acetyltransferase complex CBP/P300. HBx-mediated recruitment of CBP/P300 complex promotes transactivation activity, resulting in an acetylated (active) histone state of the target cellular genes [23–26,47]. Interestingly, as the HBV genome remains in a minichromosomal structure (bound with histone and non-histone proteins) in the infected hepatocytes, HBx also regulates replication and transcription of both the viral genome and genes by influencing the epigenetic (acetylation) state of the histone molecules in the viral minichromosomes [4,19,48]. A recent study, perhaps surprisingly, revealed a histone deacetylase (HDAC) as a direct HBx-interacting partner, explaining its transrepressive activities [26]. However, it also raises the question of whether HBx associates with other members of the cellular epigenetic machinery and what governs these interactions (Fig. 2). The time gap observed between the infection with HBV and the actual onset of HCC indicates that interaction between HBx and epigenetic machinery must play an important role in this process of gradual transformation and may be modulated by the cellular state and further exposures to different HCC risk factor(s) [4,19].

In most cases, the effects of histone modifications are more complex and could only be explained by crosstalk between them, wherein specific combinations of histone modifications affect the accumulation and function of the histone-modifying enzymes, DNA repair factors, transcription factors and chromatin remodel-
ing complexes. These interactions/crosstalk play an important role in collectively regulating the critical chromatin functions and thus avoiding genomic instability and oncogenic transformation. Cross-talk between histone modifications could be a result of modifications occurring on the same residue (in cis), on the same histone (in trans), or on different histones (in trans) [8-10,49]. At present there are no conclusive reports providing unequivocal support for HCC-specific histone modification crosstalk. However, it remains possible that different risk factor exposures and environmental cues may affect expression of different histone modification machineries and as a result different histone modifications or crosstalk between different histone modifications may be modulated, resulting in HCC initiation and progression.

5. Epigenetics-based therapy for HCC

The most recent mechanism of epigenetic inheritance involves RNAs, which in the form of either microRNAs (miRNAs) or long noncoding RNA (lncRNA) or long noncoding RNA (long ncRNA or IncRNAs) can alter gene expression states in a heritable manner. miRs are a class of small RNA molecules that regulate gene expression [8,12,32]. Their activity is a result of duplex formation between the miR and the 3’ untranslated region (UTR) of target mRNAs. This results in translational silencing by either mRNA degradation or translation blocking. Several recent studies have implicated miR alterations in different steps of hepatocellular carcinoma development and metastatic progression (Table 1). In HCC several miRs are deregulated, some of which function as oncogenes by inhibiting apoptosis (miR-221), promoting cell invasion (miR-9), or silencing c-Met/upon-expression, thereby inhibiting migration and proliferation (miR23b). miR-101, 195, 122 and-338 have a tumor suppressor gene-like function and are silenced in HCC. Down-regulation of miR-122 has been reported to correlate with suppression of the hepatic phenotype and gain of metastatic properties. miR-152, miR-602 and miR-143 show HBV infection-specific expression and regulate important cellular genes including DNMT1, RASSF1A and FNDC3B affecting pathways related to cell death, DNA damage, recombination, and signal transduction [15,27,29,30]. miR-122 and miR-196 show HCV infection-specific expression and regulate HMOS1/Bach1 and HCV genome expression [34,35]. These findings suggest that miRs could become novel molecular targets for HCC treatment [8,32,50].

In addition to the well-characterized microRNAs, long ncRNAs are also emerging as important regulatory molecules in tumor-suppressor and oncogenic pathways. Recent studies have provided mechanistic insight into long ncRNAs that regulate key cancer pathways at a transcriptional, post-transcriptional and epigenetic level. For example, it is now well established that some long ncRNAs such as XIST, HOTAIR, AIR and KCNY1OT1 interact with chromatin-remodeling complexes targeting them to specific genes to exert their functions [32,50]. The oncogenic long ncRNAs may thus hijack the epigenetic machinery to reshape the epigenetic landscape leading to cancer.

6. Conclusions and future directions

Aberrant epigenetic events play an important role in the onset and progression of hepatocellular carcinoma, and have been associated with all HCC subtypes. At the cellular level, aberrant epigenetic events influence critical cellular events (i.e., gene expression, DNA repair and cell cycle), which are further modulated by risk factor exposures and thus define the severity/ subtype of HCC. The epigenetic events (DNA methylation, histone modifications and non-coding RNAs), being specific to different risk factor exposures, form an epigenetic signature with the potential to serve as an important biomarker for early detection and prevention of HCC [12,32,42]. However, although different epigenetic events have been described/studied separately, all epigenetic events are likely interdependent and collectively define the epigenetic state of an individual cell in a given tissue for each individual. Examples of the crosstalk between different epigenetic modifications and their roles in the regulation of cellular processes and tumorigenesis are becoming more evident and remain important for a better mechanistic understanding and development of epigenetics-based therapeutic regimes.

Significant progress in the field of cancer epigenetics has enhanced our understanding of the molecular mechanisms in different cellular processes and in abnormal events involved in carcinogenesis. Unlike genetic events, epigenetic events are reversible, and thus hold better promise for therapeutic interventions. Epigenetic changes are present in almost every HCC subtype, and different HCCs harbor specific ‘epigenetic signatures’. Recent identification of DNA methylation in the HBV genome suggests that it might be an important mechanism regulating transcription and replication of the HBV virus [8,52]. However, the exact impact of the observed methylation on the function of the HBV genome remains unknown. Furthermore, it would be of interest to investigate possible epigenetic mechanisms by which HBV infection and integration of the viral genome may promote hepatocarcinogenesis (Fig. 2). Future studies should also address whether epigenetic changes induced by HBV and/or HCV infection promote HCC development by inducing direct deregulation of epigenetic states or if these changes are consequences of the activation of inflammatory pathways. In order to enable the development of new cancer therapies, more systematic studies, involving different HCC subtypes at the genomic, epigenomic and transcriptomic levels are necessary. A large number of genome-wide methods and recent developments in novel sequencing technologies should probably make these studies feasible in the next few years.
Conflict of interest

Neither of the authors or the authors’ institutions has a financial or other relationship with other people or organizations that may inappropriately influence the authors’ work or this review.

Acknowledgments

The work in the Epigenetics Group at the International Agency for Research on Cancer (Lyon, France) is supported by grants from l’Agence Nationale de Recherche Contre le Sida et Hepatites Virales (ANRS, France), l’Association pour la Recherche sur le Cancer (ARC), France; and the Ligue Nationale (Française) Contre le Cancer, France (to Z.H.). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

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Z. Herceg, A. Paliwal / Mutation Research 727 (2011) 55–61


