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Study of The Impact of DNA Methylation on the Expression of Cell Cycle Regulators in Bladder Carcinoma

KEYWORDS: Bladder cancer, Cyclin D, Cyclin E, DNA methylation.

Mohamed Abd-Ellatif	Raymonde H. Assaf	Fagr B. El-Shahat
Medical Biochemistry Department, Faculty of Medicine - Mansoura University, Mansoura, Egypt., Department of Clinical Biochemistry, College of Medicine, King Khalid University, Abha, KSA.	Medical Biochemistry Department, Faculty of Medicine - Mansoura University, Mansoura, Egypt.	Medical Biochemistry Department, Faculty of Medicine - Mansoura University, Mansoura, Egypt

Ahmed B. Shehab El-Din	Salwa M. Abou El-Khair
Urology and Nephrology Center, Faculty of Medicine - Mansoura University, Mansoura, Egypt.	Medical Biochemistry Department, Faculty of Medicine - Mansoura University, Mansoura, Egypt.

ABSTRACT *The aim of this work is to evaluate the DNA methylation status and expression of different cell cycle regulators during multistage carcinogenesis of rat urinary bladder. Materials and Methods: This experimental study included 64 male (fisher 344) rats (6 weeks old). They were divided into experimental group (n= 48) and control group (n= 16). Urinary bladder carcinogenesis was induced in rats of the experimental group by N-Ethyl N-Butane Nitrosamine (EBN) in their drinking water for 10 weeks. At the 10th, 14th, 20th, and 24th week from the time of induction of carcinogenesis, rats were sacrificed. Pathological and normal urinary bladder tissue specimens from experimental and control rats were excised and divided into 3 samples to be used for histopathological analysis, the expression of both cyclin D1 and E, and for methylation changes. The results of our study showed that the expression of both cyclin D1 and cyclin E were very highly significantly increased in hyperplasia, dysplasia, and carcinoma compared with the control group. There was a gradual methylation changes during urinary bladder carcinogenesis. Conclusion: Multiple stepwise progressive changes in methylation patterns are observed during EBN induced rat urinary bladder carcinogenesis. These methylation changes occur early during oncogenesis and are detectable prior to the appearance of clinically evident tumor. Overexpression of cyclin D1 and cyclin E is an important and frequent molecular event, at relatively early stage during the development of urinary bladder cancer. Both cyclin D1 and E could become useful biomarkers to identify precancerous lesions in the early stage of urinary bladder cancer.*

INTRODUCTION:

Carcinogenesis is a stepwise process of accumulation of genetic and epigenetic abnormalities that can lead to cellular dysfunction [1]. It has become clear that epigenetic changes are equally important for this multistep process to produce its results [2].

Methylation of CpG sites in the genome, which is generally conserved during cell replication, is considered to play important roles in cell differentiation and carcinogenesis [3]. DNA methylation is believed to be an on-off switch in gene expression. Changes in the methylation profile of the promoters represent an alternative to genetic lesions as causative factors for the tumor-specific aberrant expression of the genes [4],[5]. Alternatively, the gain of methyl groups, particularly in CpG islands [GC-rich regions of the genome, ~1 kb in length, originally characterized by their lack of methylation], is linked to the transcriptional repression of the associated genes [6], [7]. So, DNA methylation plays a key role in the regulation of gene expression, and failure to maintain normal patterns of methylation often contributes to carcinogenesis [3], [8].

Cyclin D1 is an oncogene which is related to the regulation of cell cycle. Cyclin D1 serves as a key sensor and integrator of extracellular signals in early to mid G1 phase. It is thought to move cells from G_1S and participate in the transduction of external mitogenic signals to other components of G_1/S transition cell cycle machinery, thus moving G_0 cells into G_1, and early G_1 cells into the G_1/S transition [9]. The cyclin D1 gene product is a key protein which makes cells enter into proliferation condition and cyclin D1 and CDK4 are composed of a complex which could functionally inhibit tumor suppressor gene p16 and retinoblstoma (pRb) activity [10],[11]. Overexpression of cyclin D1 has been implicated in the malignant transformation of a variety of human cancers, including bladder carcinoma [12].

Cyclin E is essential for progression through the G1-phase of the cell cycle and initiation of DNA replication by interacting with and activating its catalytic partner, the Cdk2 [13].

An extensive examination of the differences in DNA methylation state (both gains and losses) at different stages of cancer development will lead to a clearer understanding of the mechanisms involved in carcinogenesis and identification of the earliest changes in methylation will provide useful biomarkers in cancer diagnosis and treatment [6].

The aim of this work was to evaluate the DNA methylation status and expression of different cell cycle regulators during multistage carcinogenesis of rat urinary bladder.

Materials and Methods:

This experimental study was carried out at Medical Biochemistry Department and Mansoura Urology and Nephrology Center, Faculty of Medicine, Mansoura University,

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Egypt.

A. Animals:
A total of 64 male (fisher 344) rats (6 weeks old) were obtained from and housed in the animal house of Mansoura Urology and Nephrology Center. They were divided into experimental group and control group. Urinary bladder carcinogenesis is induced in experimental group (n= 48). They were given 0.05% N. ethyl N- butan (4.O.L) nitrosamine (EBN) and 1.34% Nacl in their drinking water for 10 weeks then they were maintained on the basal diet and tap water without any treatment till the time of scarification [14], [15].

All rats were housed four per cage in an animal facility and a controlled condition with at a temperature of 22 ± 2 °C and 55 ± 5% relative humidity. The rats were given free access to tap water and food and were observed daily, their body weights were measured weekly throughout the duration of the experiment. Stop gaining weight and then loss of weight usually began to occur at the 13th or 14th week. Caschexia was marked from the 18th week. Eight rats died during the experiment. Sex and age matched control rats (n=16) were given free access to tap water and basal diet without any treatment till the time of scarification.

B. Tissue sampling
At the 10^{th}, 14^{th}, 20^{th}, and 24^{th} week from the time of induction of carcinogenesis, rats were sacrificed; urinary bladders were removed and examined. Pathological and normal urinary bladder tissue specimens from experimental and control rats were excised and divided into 3 samples.

- 1^{st} sample was fixed in 10% formalin, sectioned, and stained with Hematoxylin and Eosin (H&E) for histopathological analysis. *According to the histopathological finding, the rats were divided into four groups: Group I: hyperplasia group (n= 13), included simple and papillary hyperplasia. Group II: dysplasia group (n= 12), included papillary hyperplasia with mild and moderate dysplastic changes. Group III: carcinoma group (n=15), included carcinoma in situ and papillary transitional cell carcinoma grade I and II. Group IV: control group (n=16), included rats with normal bladder.*

- 2^{nd} sample was snap frozen in liquid nitrogen and immediately analyzed for expression of cyclin D1, cyclin E.

- 3^{rd} sample was immediately stored at -20 °C until it was analyzed for methylation changes by DNA extraction and methylation sensitive arbitrarily primed polymerase chain reaction (AP-PCR).

C. Methodology:
(A) Assay of expression of cyclin D1 mRNA and cyclin E mRNA:

Total RNA extraction from urinary bladder tissue specimens. E.Z.N.A Total RNA kit for total RNA purification from tissues provided by Omega Bio-tek USA Cat. No R 6936-01was used.

RT-PCR of the extracted RNA was performed using Ready-to-Go RT-RCR beads for first strand cDNA synthesis and PCR reactions provided by Amersham Biosciences, England, cat. No. 27-9266-01.

Gene-specific primers (Amersham Biosciences):

Cyclin Dl primers: Their sequences were as follow: (sense) 5`-TGA CAA CTC TAT CCG CCC CGA-3` and (antisense) 5`-GAA AGT GCG TTG TGC GGT AGC-3`.

Cyclin E primers: Their sequences were as: (sense) 5` CTG GCT GAA TGT TTA TGT CC-3` and (antisense) 5` TCT TTG CTT GGG CTT TGT CC-3`.

- Cyclin D1 produced a single 314 bp PCR product.

- Cyclin E produced a single 392 bp PCR product.

- The images were digitalized, recorded and quantitated with image analysis software (Scion Image, Scion Corporation, USA) on a personal computer. Peak intensity of the PCR product derived from the target gene was divided over HPRT PCR product, the internal control for RT- PCR. This relative ratio of the target gene divided on HPRT represent the relative expression of the target gene. Each target gene expression was evaluated by comparison of the results with those of normal controls at each experimental stage.

(B) Detection of DNA methylation changes by methylation-sensitive arbitrarily primed polymerase chain reaction (AP-PCR):

DNA extraction from urinary bladder tissue specimen: Using E.Z.N.A. Tissue DNA kits for DNA purification from tissue provided by Omega Bio-tek USA Cat. No. 3496-01.

Restriction enzyme digestion of genomic DNA: for each DNA sample, 2 restriction digests were performed: one with RsaI and MspI and the other with RsaI and HpaII (Roche Molecular Biochemicals, Indianapolis, IN). RsaI is methylation-insensitive, while MspI and HpaII are methylation-sensitive.

Both MspI and HpaII cut between cytosine residues at 5`-CCGG -3` sites. MspI would not cut if the external cytosine is methylated, and HpaII would not cut if the internal cytosine is methylated whereas MspI is insensitive to the methylation status of the internal cytosine [16]. This double restriction enzyme was used to reduce the number of PCR fragments and potential artifacts that might be amplified in the AP-PCR.

Methylation – sensitive AP- PCR:

The methylation-sensitive AP-PCR is highly dependent on the sequence of the arbitrary primer. The GC-rich primers are more successful at amplifying GC- rich DNA fragments because there is a high probability that they preferentially anneal to sequences associated with CpG islands. The sequences of the arbitrary single primer used were as follow: 5` AAC CCT CAC CCT AAC CCC GG-3`.

AP-PCR: Using PuRe TaqTM Ready-to-Go polymerase chain reaction (PCR) Beads for performing PCR amplifications (Amersham Biosciences Cat. No. 27-9558-01).

D. Statistical analysis:
All data were analyzed using statistical package for social science program (SPSS version 13.0). The results were expressed as the mean ± SD. Measurement data were analyzed using one-way analysis of variances (ANOVA). When the distribution of data was nonparametric, Kruskal Wallis H test was used for comparison between more than two groups and Mann-Whitney (unpaired Wilcoxon) test was used for comparison between two groups. Spearman rank

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and Pearson correlation coefficient were used to find the degree of correlation between the different variables. P value ≤ 0.05 was considered significant.

Results:
The results of our study showed that cyclin D1 expression was very highly significantly increased in hyperplasia, dysplasia, and carcinoma compared with the control group (figures 1&2).

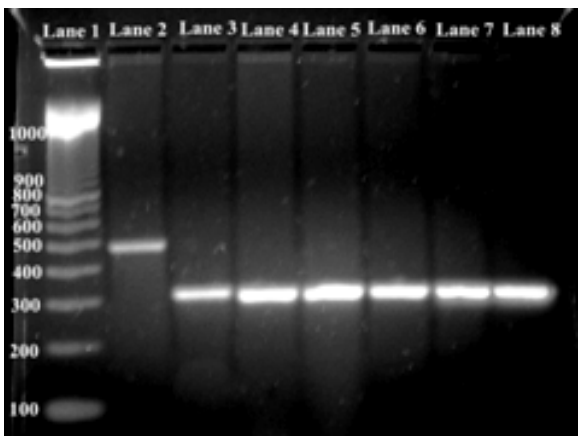

Fig. 1- RT- PCR products using specific primers for cyclin D1. Lane1: DNA marker, Lane2: RT-PCR product of HPRT (the internal control), Lane3: normal mucosa sample, Lane4: simple hyperplasia sample, Lane5: papillary hyperplasia sample, Lane6: hyperplasia with moderate dysplastic changes sample, Lane7: carcinoma in situ sample, Lane8: transitional cell carcinoma sample.

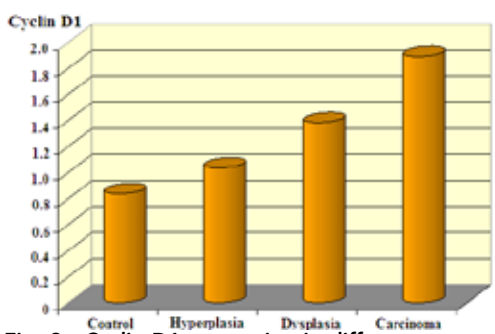

Fig. 2- Cyclin D1 expression in different groups

This study, also, revealed that cyclin E expression was very highly significantly increased in hyperplasia, dysplasia, and carcinoma compared with the control group (figures 3&4).

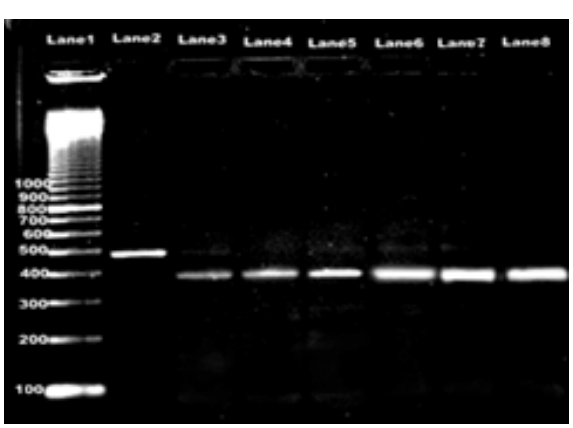

Fig. 3- RT- PCR products using specific primers for cyclin E . Lane1: DNA marker, Lane2: RT-PCR product of HPRT(the internal control), Lane3: normal mucosa sample, Lane4: simple hyperplasia sample, Lane5: papillary hyperplasia sample, Lane6: in papillary hyperplasia with moderate dysplastic changes sample, Lane7: in carcinoma in situ sample, Lane8: transitional cell carcinoma sample.

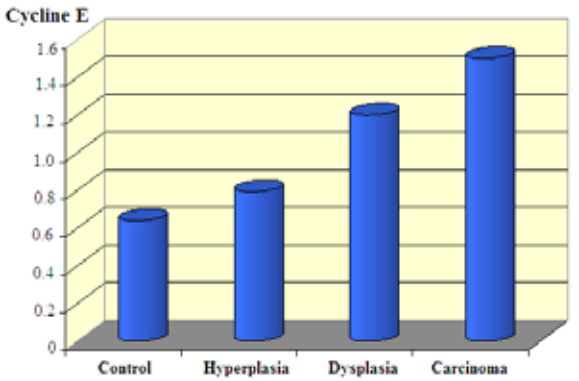

Fig. 4- Cyclin E expression in different groups.

The overexpression of both cyclin D1 and cyclin E may be indicative that cyclin D1 and cyclin E may cooperate in driving proliferation in early stages of tumor progression.

Detection of methylation changes:
In this experimental study, the patterns of bands of PCR product images were examined to determine the methylation status at external and internal cytosines at 5`-CCGG-3` sequences. Segments of DNA between or at sites of primer annealing are amplified by PCR unless a site within the region is cut with HpaII or MspI. Thus, bands seen in both MspI and HpaII digest lanes are indicative of the presence of methylated 5`-CCGG-3` sites. Bands present in HpaII digest lanes but not in MspI digest lanes represent methylation of the internal cytosine of 5`-CCGG- 3` site. Conversely, bands seen more prominently in MspI digest lanes are indicative of methylation of the external cytosine.

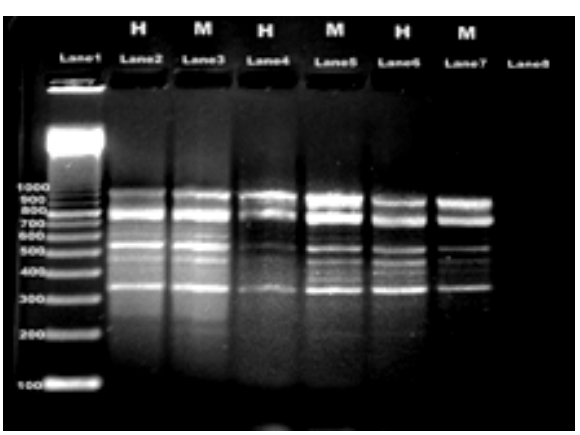

Fig. 5- This figure revealed the methylation- sensitive AP-PCR of normal and pathological samples of dysplasia group using a single primer in the AP- PCR after a 16 hours restriction enzyme digestion with 5 units of Rsa I + Hpa II or Rsa I + Msp I (H: Rsa I + Hpa II digested DNA, M: Rsa I + Msp I digested DNA). Lane1: DNA Marker, Lane2 & Lane3: normal DNA from matched control rat of control group, Lane4 & lane5: DNA sample from a rat of dysplasia group, Lane6 & lane7: DNA sample from another rat of dysplasia group.

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The results of our study revealed a gradual and important methylation changes during urinary bladder carcinogenesis. In hyperplasia stage there was a moderate change in DNA methylation in the form of moderate hypomethylation in both internal and external cytosine. However, in dysplasia samples the banding pattern was more markedly different as compared to the control samples. As regards the carcinoma stage, a large amount of methylation change was present; there was a large hypomethylation at both internal and external cytosine together with hypermethylation at external cytosine (figures 5&6). The changes in methylation pattern in cancer, as detected in our study, may be related to changes in levels and activity of certain methyltransferases.

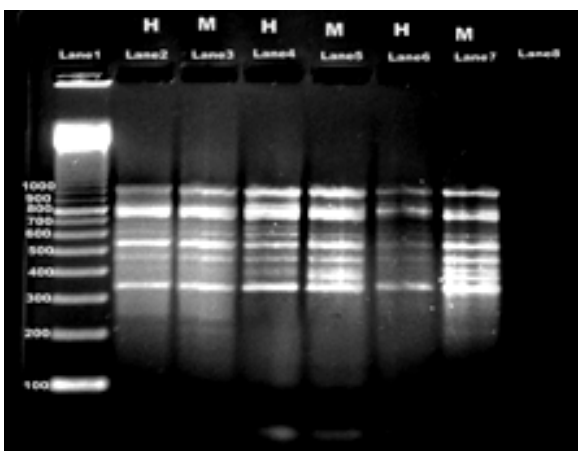

Fig. 6- This figure revealed that in carcinoma group of samples, a large amount of methylation change is present. There are 6-10 different bands in carcinoma samples in comparison with control samples (H: Rsa I + Hpa II digested DNA, M: Rsa I + Msp I digested DNA). There are 5-8 bands seen in the RsaI/MspI and RsaI/HpaII lanes of control samples but not or less distinctly seen in the carcinoma samples. This is indicative of a moderate and large amount of hypomethylation at the external and internal cytosine in carcinoma samples. Also, there are 1-3 bands that are seen in the carcinoma samples but not seen in the control samples. This is indicative of a small amount of hypermethylation at the external cytosine sites of carcinoma samples.

Discussion:

The detection of methylation changes associated with oncogenic transformation is of critical importance in understanding how DNA methylation may contribute to oncogenesis [5],[17]. Regulation of the cell cycle is also important for normal cell growth and differentiation. Disorders of the cell cycle regulatory machinery play a key role in the pathogenesis of cancer [12]. Rat urinary bladder cancer induced by N. ethyl N. butan (4.O.L) nitrosamine (EBN) has morphological, biological and molecular parallels to human bladder cancer [18].

In this experimental study we investigated cyclin D1 expression in different histopathological stages of urinary bladder carcinogenesis. There was a highly significant difference in cyclin D1 expression in the different histopathological groups. Cyclin D1 expression was a highly significantly increased in hyperplasia, dysplasia, and carcinoma compared with the control group.

The overexpression of cyclin D1, detected in our study, may be due to its transcriptional activation. Cyclin D1 has a very short half life of about 20 minutes and its expression is generally growth factor inducible [19], and in most cancer types, cyclin D1 overexpression results from induction by oncogenic signals rather than a clonal somatic mutation or rearrangement in the cyclin D1 gene [20]. So, the overexpression of cyclin D1 in the preneoplastic lesions and carcinomas is propably related to the acquisition of autonomous growth properties.

The cyclin D1 promoter is composed of a CpG island that can be potentially regulated by DNA methylation and that DNA hypomethylation may participate in the long-distance deregulation of cyclin D1 gene observed in malignancies [21]. The steady state expression of the cyclin D1 gene was influenced by the methylation status of the 5`-flanking region in rat leukemic and normal endometrial stromal cells [22]. Aberrant methylation of the CpG loci within the 5`-regulatory regions play a principle role in tissue specific expression of genes by affecting the interactions of DNA with chromatin proteins and transcription factors [21]. This is in agreement with the results of our study as we demonstrated a DNA hypomethylation in preneoplastic and tumor tissues which may be an explanation for the cyclin D1 overexpression observed in different stages of bladder carcinogenesis.

Also, this overexpression of cyclin D1 may provide a growth advantage for the preneoplastic lesions (making them more likely to progress to advanced lesions such as papillomas or carcinomas) and for the tumors under the conditions of the current bladder carcinogenesis protocol. This suggestion supports the previous studies of *Kim and Zhao* [9] and, *Elliman et al* [11], that reported that cyclin D1 serves as a key sensor and integrator of extracellular signals in early to mid G1 phase, mediating its function through binding the Cdks, histone acetylase and histone deacetylases to modulate local chromatin structure around the genes that are involved in regulation of cell proliferation and differentiation.

Cyclin D1 overexpression during urinary bladder carcinogenesis was also reported [14], [24], [25] and there was more than two folds increase in cyclin D1 mRNA expression during rat bladder carcinogenesis [23]. Overexpression of cyclin D1 was also demonstrated in different models of rat carcinogenesis including colon [25], fore stomach [27] and breast [28] oncogenesis.

In our experimental study we investigated cyclin E expression in different histopathological stages of urinary bladder carcinogenesis. There was a highly significant difference in cyclin E expression in different histopathological groups. Cyclin E expression was highly significantly increased in hyperplasia, dysplasia, and carcinoma compared with the control group. In agreement with our results, cyclin E overexpression during urinary bladder oncogenesis was also reported by *Lee et al* [23], and *Khan et al* [24].

The increase in expression of cyclin E, detected in our study, may be explained by induction of its expression at the transcriptional level. As cyclin E has a major role in the control of the G1- and S-phase transitions, its expression is tightly regulated both at the transcriptional level and by ubiquitin-mediated proteolysis [13]. Also, overexpression of cyclin D1, which was proved in our experimental study, increases cyclin E expression. Cyclin D/Cdk protein kinase complexes phosphorylate pRb, releasing E2F from pRb. This phosphorylation allows the accumulation of E2F1, E2F2, and E2F3a that activate the transcription of a large

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number of genes essential for DNA replication as well as further cell cycle progression [29], [30]. Cyclin E gene is included among these genes [31].

Cyclin E is involved in early events and control of DNA replication during S phase. Ectopic expression of cyclin E affects the G1/S transition resulting in an uncoupling of DNA replication and cell cycle progression and affecting the fidelity of DNA replication [32]. Therefore, overexpression of cyclin E plays an important role in tumour progression. Cyclin E overexpression has been reported in colon [26], breast [32] and ovarian [33] carcinogenesis. Our results revealed overexpression of both cyclin D1 and cyclin E with a significant positive correlation between them in carcinoma group. These results support the study of *Khan et al.* [24] who reported overexpression of both cyclin D1 and cyclin E during cancer bladder progression. Our results,also, are indicative that cyclin D1 and cyclin E may cooperate in driving proliferation in early stages of tumour progression.

DNA methylation is a dynamic but tightly regulated process. Methylation patterns are faithfully transmitted to the next generations during cell division. With the noted exceptions of imprinted genes and several genes on the inactive X chromosome in females, CpGs within CpG islands are normally unmethylated while most CpGs outside CpG islands are methylated. It has been suggested that these patterns of methylation may serve to compartmentalize the genome into transcriptionally active and inactive zones [7], [34], [35]. In the present study, patterns of DNA methylation of the genome were investigated during stages of urinary bladder carcinogenesis in a rat model. Matched normal and preneoplastic or tumor samples were compared to identify differential methylation. The results of our study revealed a gradual and important methylation changes during urinary bladder carcinogenesis. In hyperplasia stage there was a moderate change in DNA methylation in form of moderate hypomethylation in both internal and external cytosine. However, in dysplasia samples the banding pattern was more markedly different as compared to the control samples. There was both hypomethylation and hypermethylation changes at both internal and external cytosine. As regards the carcinoma stage, a large amount of methylation change was present. There was a large hypomethylation at both internal and external cytosine together with hypermethylation at external cytosine. DNA methylation alterations occur throughout all stages of carcinogenesis and that methylation patterns in tumor cells are altered relative to those of normal cells [35]. A global hypomethylation of the genome is accompanied by region-specific hypermethylation [36]. This global hypomethylation occurs in repetitive elements localized in satellite sequences or centromeric regions [37], and in specific oncogenes leading to its activation [38]. Furthermore, hypomethylation of non-promoter regions may lead to a decreased stability of the genome due to an increase in the expression of transposons that are typically silenced by methylation [39]. However, hypermethylation occurs in promoters of several tumor suppressor genes, including p16 [40] and retinoblastoma gene [41]. This hypermethylation is an alternative mean for the inactivation of the tumor suppressor genes in cancer [42]. Maintaining normal patterns of methylation is dependent on multiple, interdependent factors including maintenance and de novo methylation, demethylation that is not linked to DNA replication, the availability of methyl group sources (S-adenosyl methionine is the proximate methyl donor), cellular proliferation, and cellular differentiation [43]. Alteration of one or more of these factors may lead to hyper- and/or hypomethylation, both of which have been shown to contribute to carcinogenesis [3].

The changes in methylation pattern in cancer, as detected in our study, may be related to changes in levels and activity of certain methyltransferases. DNA methyltransferases are responsible for maintenance of methylation at CpG and non CpG sites. When treated with certain tumour promoters, specific methylases may be more or less affected than others leading to differences in the level of methylation change at the CpG and CpCpG sites [8], [44]. This pattern of methylation changes that was detected in our study, in which both hypomethylation and hypermethylation contribute significantly to bladder carcinogenesis, was also reported during skin carcinogenesis [3], [45] and during liver oncogenesis [44]. The results of our study supported the previous studies [17], [46] that demonstrated the same pattern of methylation changes, including both hypomethylation and hypermethylation, in human urinary bladder cancer. DNA methylation control of gene expression plays an important role in the development of cancer [35]. The results of our study indicate that alterations in DNA methylation may play a variety of roles in carcinogenesis.

Conclusion:

Multiple stepwise progressive changes in methylation patterns are observed during EBN induced rat urinary bladder carcinogenesis. These methylation changes occur early during oncogenesis and are detectable prior to the appearance of clinically evident tumor. Overexpression of cyclin D1 and cyclin E is an important and frequent molecular event, at relatively early stage during the development of urinary bladder cancer. Both cyclin D1 and E could become useful biomarkers to identify precancerous lesions in the early stage of urinary bladder cancer.

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REFERENCE

[1] W.E. Farrell: Epigenetic mechanisms of tumorigenesis. Horm. Metab. Res. Jun (2005); 37 (6): 361-368. | [2] P.A. Jones: Epigenetics in carcinogenesis and cancer prevention. Ann. N. Y. Acad. Sci. Mar (2003); 983: 213- 219. | [3] R.E. Watson, G.M. Curtin, D.J. Doolittle and J.I. Goodman: Progressive alterations in global and GC-rich DNA methylation during tumorigenesis. Toxicological Sciences. Oct (2003); 75 (2): 289-299. | [4] J. Yu, M. Ni, J. Xu, H. Zhang, B. Gao, J. Gu, J. Chen, L. Zhang, M.Wu and J. Zhu: Methylation profiling of twenty promoter CpG islands of genes which may contribute to hepatocellular carcinogenesis. B.M.C. Cancer. Nov (2002); 15; 2 (1): 29- 39. | [5] D. H. Lim and E. R. Maher "DNA methylation: a form of epigenetic control of gene expression" The Obstetrician & Gynaecologist., 2010; 12:37–42. | [6] J.R. Graham, H.H. Tim, C. Chuan-Mu and J.J. Keith: A novel technique for the identification of CpG islands exhibiting altered methylation patterns (ICEAMP). Nucleic Acids Research. 2001; 29 (24) el 23. | [7] S. Aktas, P. Erçetin, A. Celebiler and G. Diniz "Methylation Profile of the Promoter CpG Islands of Death Associated Protein-Kinase in Nephroblastoma" Journal of Pediatric Oncology, 2013, 1, 83-87. | [8] J. R. Wagner, S. Busche, B. Ge, T. Kwan, T. Pastinen* and M. Blanchette "The relationship between DNA methylation, genetic and expression inter-individual variation in untransformed human fibroblasts" Genome Biology., 2014, 15:R37. | [9] Y.T. Kim and M. Zhao: Aberrant cell cycle regulation in cervical carcinoma. Yonsei Medical Journal. 2005; 46 (5): 597-613. | [10] Z. Lian-Fu, L. Pei-Zhong, Q. Fing-Ying, G. Jian-Wen and L. Jiang-Hui: Flow cytometric analysis of DNA, telomerase content and multi-gene expression in esophageal epithelial dysplasia. World J Gastroenterol. November (2003); 9 (11): 2409-2412. | [11] S.J. Elliman, B.V. Howley, D.S. Mehta, H.O. Fearnhead, D.M. Kemp and L. R. Barkle "Selective repression of the oncogene cyclin D1 by the tumor suppressor miR-206 in cancers" Oncogenesis (2014) 3, e113; doi:10.1038/oncsis.2014.26. | [12] K.E. Bijwaard and J.H. Lichy: Determination of cyclin D1 expression by quantitative real-time, reverse-transcriptase polymerase chain reaction. Methods Mol. Med. 2004; 277-296. | [13] S. Mazumder, E.L.Dupree and A. Almasan: A dual role of cyclin E in cell proliferation and apoptosis may provide a target for cancer therapy. Curr. Cancer Drug Targets. February (2004); 4 (1): 65-75. | [14] K. Takaba, K. Saeki, K. Suzuki, H. Wanibuchi and S. Fukushima: Significant overexpression of metallothionein and cyclin D1 and apoptosis in the early process of rat urinary bladder carcinogenesis induced by treatment with N-butyl-N-(4-hydroxybutyl) nitrosamine or sodium L-ascorbate. Carcinogenesis. April (2000); 21 (4): 691-700. | [15] S.M. Cohen, L.L. Arnold, M. Cano, M. Ito, E.M. Garland and R.A. Shaw: Calcium phosphate containing precipitate and the carcinogenicity of sodium salts in rats. Carcinogenesis. Apr (2000); 21 (4): 783-792. | [16] M.B. Mann and H.O. Smith: Specificity of Hpa II and Hae III DNA methylases. Nucleic Acids Res. 1977; 4: 4211-4221. | [17] G. Liang, M.L. Gonzalgo, C. Salem and P.A. Jones: Identification of DNA methylation differences during tumorigenesis by methylation sensitive arbitrarily primed polymerase chain reaction. Methods. 2002; 27: 150-155. | [18] I. Ariel, S. Ayesh, O. Gofri, B. Ayesh, R. Abdul-Ghani, et al: Gene expression in the bladder carcinoma rat model. Mol. Carcinog. Oct (2004); 41 (2): 69-76. | [19] C.J. Sherr: The Pezcoller lecture: cancer cell cycles revisited. Cancer Res. 2000; 60: 3689–3695. | [20] M. Fu, C. Wang, Z. Li, T. Sakamaki and R.G. Pestell: Minireview: cyclin D1: normal and abnormal functions. Endocrinology. 2004; 145 (12): 5439-5447. | [21] H. Liu, J. Wang and E.M. Epner: Cyclin D1 activation in B-cell malignancy: association with changes in histone acetylation, DNA methylation and RNA polymerase II binding to both promoter and distal sequences. Blood. October (2004); 104 (8): 2505-2513. | [22] S. Kitazawa, R. Kitazawa and M. Sakan: Transcriptional regulation of rat cyclin D1 gene by CpG methylation status in promotor region. J. Biol. Chem. 1999; 274 (40): 28787-28793. | [23] C.C. Lee, S. Yamamoto, H. Wanibuchi, S. Wada, K. Sugimura, T. Kishimoto and S. Fukushima: Cyclin D1 overexpression in rat two stage bladder carcinogenesis and its relationship with oncogenes, tumor suppressor genes and cell proliferation. Cancer Res. November (1997); 57 (21): 4765-4776. | [24] A.A. Khan, P.D. Abel, K.S. Chaudhary, Z. Gulzar, G.W.H. Stamp and E.N. Lalani: Inverse correlation between high level expression of cyclin E and proliferation index in transitional cell carcinoma of the bladder. Mol. Pathol. December (2003); 56 (6): 353-361. | [25] E. Ioachim, M. Michael, N.E. Stavropoulos, E. Kitsiou, K. Hastazeris, et al: Expression patterns of cyclins D1, E and cyclin-dependent kinase inhibitors p21 (Waf1/Cip1) and p27 (Kip1) in urothelial carcinoma: correlation with other cell cycle related proteins (Rb, p53, ki-67 and PCNA) and clinicopathological features. Urol. Int. 2004; 73 (1): 65-73. | [26] K. Hur, J.R. Kim, B. Yoon, J.K. Lee, J.H. Choi, G.T. Oh and D.Y. Kim: Overexpression of cyclin D1 and cyclin E in 1, 2 dimethylhydrazine dihydrochloride induced rat colon carcinogenesis. Journal of Veterinary Science. 2000; 1 (2): 121-126. | [27] M. Kaneko, K. Morimura, T. Nishikawa, H. Wanibuchi, N. Takada, H. Osugi, H. Kinoshita and S. Fukushima: Different genetic alterations in rat forestomach tumors induced by genotoxic and non genotoxic carcinogens. Carcinogenesis. October (2002); 23 (10): 1729-1735. | [28] S.J. Weroha, S.A. Li, O. Tawfik and J.J. Li: Overexpression of cyclins D1 and D3 during estrogen induced breast oncogenesis in female ACI rats. Carcinogenesis. Mar (2006); 27 (3): 491-498. | [29] J.W. Harbour and D.C. Dean: The Rb/E2F pathway: expanding roles and emerging paradigms. Genes Dev. 14: 2393–2409. | [30] M. Scaltriti, P. J. Eichhorn, J. Cortés, L. Prudkin, C. Aura, J. Jiménez, S. Chandarlapaty et al "Cyclin E amplification/overexpression is a mechanism of trastuzumab resistance in HER2+ breast cancer patients" PNAS., March 1, 2011 vol. 108 No. 9 3761–3766. | [31] N. Dyson: The regulation of E2F by pRB-family proteins. Genes Dev. 1998; 12: 2245-2262. | [32] T. Lindahl, G. Landberg, J. Ahlgren, H. Nordgren, T. Norberg, et al: Overexpression of cyclin E protein is associated with specific mutation types in the p53 gene and poor survival in human breast cancer. Carcinogenesis. March (2004); 25 (3): 374-380. | [33] K.K. Kim, J.C. Shim and J.R. Kim: Overexpression of p21, cyclin E and decreased expression of p27 in DMBA (7, 12-dimethylbenzanthracene) induced rat ovarian carcinogenesis. Path. Int. May (2003); 53 (5): 291-296. | [34] J.F. Costello and C. Plass: methylation matters. J. Med. Genet. 2001; 38: 285-303. | [35] J. Paluszczak and W. Baer-Dubowska: Epigenome and cancer: new possibilities of cancer prevention and therapy? Postepy Biochem. 2005; 51 (3): 244-250. | [36] P.A. Jones and P.W. Laird: Cancer epigenetics comes of age. Nat. Genet. 1999; 21: 163-167. | [37] C. Plass: Cancer epigenomics. Human Molecular Genetics. 2002; 11 (20): 2479-2488. | [38] M.J. Hoffmann and W.A. Schulz: Causes and consequences of DNA hypomethylation in human cancer. Biochem. Cell Biol. Jun (2005); 83 (3): 296-321. | [39] K.D. Robertson and P.A. Jones: DNA methylation: past, present and future directions. Carcinogenesis. 2000; 21 (3): 461-467. | [40] A.C. Patel, C.H. Anna, J.F. Foley, P.S. Stockton, F.L. Tyson, J.C. Barrett and T.R. Devereux: Hypermethylation of the p16 promoter in B6C3F1 mouse primary lung adenocarcinomas and mouse lung cell lines. Carcinogenesis. 2000; 21: 1691-1700. | [41] S.B. Baylin: DNA methylation and gene silencing in cancer. Nat. Clin. Pract. Oncol. Dec (2005); 2 (1): S4-S11. | [42] P.K. Ha and T.A. Califano: Promotor methylation and inactivation of tumor suppressor genes in oral squamous cell carcinoma. Lancet Oncol. Jan (2006); 7 (1): 77-82. | [43] J.I. Goodman and R.E. Watson: Altered DNA methylation: a secondary mechanism involved in carcinogenesis. Pharmacol. Toxicol. 2002; 42: 501-525. | [44] R.E. Watson and J.I. Goodman: Effects of phenobarbital on DNA methylation in GC-rich regions of hepatic DNA from mice that exhibit different levels of susceptibility to liver tumorigenesis. Toxicological Sciences. 2002; 68: 51-58. | [45] A.N. Bachman, G.M. Curtin, D.J. Doolittle and J.I. Goodman: Altered methylation in gene specitic and GC-rich regions of DNA is progressive and nonrandom during promotion of skin tumorigenesis. Toxicological Sciences. 2006; 91 (2): 406-418. | [46] I.D. Markle, J. Cheng, G. Liang, D. Shibata, P.W. Laird and P. Jones: Global and gene specific epigenetic patterns in human bladder cancer genomes are relatively stable in vivo and in vitro overtime. Cancer Res. August (2001); 61: 5875-5884. |