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Evaluation of cellular tumor suppressor protein p53 antigen and 5-Methylcytosine (methylated DNA) as a Diagnostic Biomarker for Hepatocellular Carcinoma in Egypt

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Abstract

Background: Hepatocellular Carcinomas (HCCs) are mostly diagnosed at later stages, necessitating new methods for recognition and investigation of HCC. However, Alpha-fetoprotein (AFP) is still an important diagnostic biomarker for HCC, the need for alternate and reliable diagnostic biomarkers are essential.

Objective: This work aimed to evaluate cellular tumor suppressor protein p53 antigen and 5-Methylcytosine (methylated DNA) as biomarkers for the diagnosis of liver cancer in patients in Egypt.

Materials and Methods: This investigation was performed on 90 patients were assigned to three groups; group I (Control group), group II (Cirrhotic group) and group III (HCC group); each contained 30 patients:

Laboratory investigations and abdominal ultrasonography were performed to diagnose liver cirrhosis. However, the HCC diagnosis was performed either percutaneous biopsy or radiologically by abdominal ultrasound and a computed tomography (CT or CAT) scan based on the guidelines of the American-Association for Liver Diseases.

AFP & protein p53 antigen and 5-Methylcytosine (methylated DNA) levels were estimated in all groups.

Results: 5-methylcytosine (5-mC) is 0.894 at cutoff (>1.3%) with a sensitivity (96.67%), a specificity (65%), a positive predictive value (57.46%), and a negative predictive value (97.06%). For the diagnosis of HCC, the area under the curve of P53 was 0.894 at cutoff ≤14.77 pg/ml with a sensitivity (83.33%), a specificity (85%), a positive predictive value (73.16%), and a negative predictive value (91.03%).

Conclusion: Combining both serum 5-mC with P53 may serve as a new biomarker for diagnosis of HCC with a higher sensitivity than AFP.

Key words: Cellular tumor antigen p53, methylated DNA 5mc%, Cirrhotic, hepatocellular carcinoma HCC

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Introduction

Hepatocellular carcinoma (HCC), a primary malignancy of the liver originating from the hepatic stem cells (Alison 2005), is a world-wide dilemma and its epidemiological data varies among patients from different geographical areas. HCC is a health challenge in Egypt. This country is the sixth major nation in Arab countries and the Middle East, the third-largest nation in Africa, and the 15th foremost populated country in the world, with over 90 million citizens. El-Zayadi and his colleagues (2005) reported in their study an almost two-fold increase of HCC in patients with a chronic liver disease over one year.

Due to the raised rate of morbidity, highly aggressive course, heigh ratio of recurrence after the intervention and resistance to traditional treatments, the 5-year survival level of patients with untreated hepatocellular carcinoma is <5%, being among cancers with the poorest prediction (Parkin et al., 2005). However, the prediction can be improved by early diagnosis, optimal treatment, and early recognition of recurrence. When diagnosed at early stage, treatment can be curative. In fact, most HCCs are diagnosed at late stages. Accordingly, there is a crucial need to improve methods to observe and evaluate individual chances of liver cancer (Sterling et al., 2012).

Alpha-fetoprotein (AFP) is the most important diagnostic biomarker for HCC. However, it has a sub-optimal diagnostic characteristic for HCC surveillance. Firstly, there is a rise in AFP level in patients with cirrhosis and chronic hepatitis (Taketa et al. 1990). Then, only a small proportion of HCC was present with a high AFP level at the early stage (10-20%) (Yamashita et al., 2008), so reliable and advanced diagnostic biomarkers are needed to complement the AFP (Shang et al., 2012).

In cancer, DNA repair genes are generally repressed through hypermethylation of the promoters of CpG islands. In neck and head squamous cell carcinomas at least fifteen DNA repair genes have repeated hypermethylated promoters gene region; these genes are PER1, HUS1, SETMAR, SPRTN, FAAP20, FANCE, GEN1, XRCC1, MLH3, PMS1, RAD51B, XRCC3, RAD54B, BRCA1, and SHFM1 (Rieke et al., 2016). In about 17 types of cancers in DNA repair genes are deficient as the result of the hypermethylation of their promoters (Bernstein and Bernstein, 2015). As an example, the promoter of MGMT DNA repair gene is hypermethylated in the half of brain cancers, 74% of thyroid cancers, 40-90% of colorectal cancers, 88% of stomach cancers, and 93% of bladder cancers. The hypermethylation of the promoter of LIG4 occurs in 82% of colorectal cancers. The NEIL1 promoter hypermethylation occurs in 42% of non-small-cell lung cancers and in 62% of neck and head cancers. The hypermethylation of the promoter of ATM occurs in 47% of non-small-cell lung cancers. The hypermethylation of the promoter of MLH1 occurs in 48% of non-small-cell lung cancer. The hypermethylation of the promoter of FANCB occurs in 46% of neck and head cancers.

However, the promoters of FEN1 and PARP1 genes, are hypomethylated and these genes have over-expression in various cancers. FEN1 and PARP1 are crucial genes DNA repair pathways. Overexpression of this pathway causes additional mutations that can cause cancer. PARP1 is expressed highly in tyrosine kinase-activated leukemia, neuroblastoma (Newman et al., 2015), testicular and other germ cell tumors (Mego et al., 2013), and Ewing's sarcoma (Newman et al., 2002). FEN1 is expressed highly in the common cancers of the breast (Singh et al., 2008), prostate (Lam, Seligson et al., 2006), stomach (Wang et al., 2014), neuroblastomas (Krause, Combaret et al., 2005), pancreatic (Iacobuzio-Donahue et al., 2003), and lung (Nikolova et al., 2009).

DNA damage is a major basic reason for cancer (Bernstein et al., 2013). If accurate repair of DNA is deficient, DNA damages accumulate. Due to error-prone translesion synthesis, such additional DNA damages increase mutational errors in DNA replication. Additional DNA damages can also heighten epigenetic alterations due to mistakes during DNA repair (Cuozzo et al., 2007). Such epigenetic alterations and mutations can lead to cancer. Thus, CpG island hyper/hypomethylation in the promoters of DNA repair genes is likely central to the advancement of cancer.

p53-mediated apoptosis primarily relies on death stimuli, which directly or indirectly target the mitochondria through the pro-apoptotic members of the Bcl-2 family, for example, Bax and Bak revealed decreased expression in HCC with mutated TP53 (Pietsch et al., 2008). The normal liver is comparatively resistant to cell death mediated by p53, and the association between apoptosis and the p53 translocation to the mitochondria after DNA damage is rarely noted (Amaral et al., 2009). In cultured HCC cells, the activation of p53 favorably initiates cell cycle arrest rather than apoptosis, and also the mitochondrial-dependent p53 apoptosis is often terminated in hepatocytes (Xue et al., 2007). One possible process responsible for this alteration is that p53 activation outcomes in the improved expression of hepatic insulin-like growth factor-binding protein-1 (IGFBP1) that stops apoptosis by antagonizing the mitochondrial p53 (Leu and George 2007). It is obvious that tumor suppressor protein p53 plays a role in DNA ploidy conservation and mitotic fidelity in hepatocytes of both regenerative and normal liver. In quiescent livers, hepatocytes exhibit greater ploidy levels in the absence of p53, and this phenotype is further overstated when the tissues withstand regeneration after partial hepatectomy (Kurinna et al., 2013). p53 not only limits malignant transformation by apoptosis but also it does so in a non-cell autonomous manner via the release of a senescence-associated secretory phenotype (SASP) to inhibit tumorigenesis by promoting a tumor-suppressive microenvironment. Ablation of the p53-dependent senescence program in hepatic stellate cells in chronic liver injury augments liver cirrhosis and fibrosis that are associated with decreased survival. Moreover, p53 damage increases the conversion of adjacent epithelial cells to hepatocellular carcinoma (Lujambio et al., 2013). In conclusion, p53 performs an important and exclusive function in HCC and normal hepatocytes, and it is necessary to additionally explore the mechanisms and alterations behind this regulation.

This study aimed at evaluating both 5-mC and p53 as biomarkers for HCC diagnosis in Egyptian patients.

Materials and Methods

The present study was done at the National Cancer Institute NCI, Cairo University Hospitals from January 2017 to August 2018. A total number of 90 patients were assigned to this investigation and assigned in three groups.

1. The control group (G1):

Thirty apparently, healthy individuals (15 males and 15 females) (nonsmoker and nonalcoholic), free from any liver, kidney, and heart diseases with the age range of 20-60 years were assigned to the study after their clinical and biochemical investigations were completed.

2. Cirrhotic group (G2):

Thirty individuals (18 males and 12 females) (nonsmoker and nonalcoholic), free from kidney and heart diseases with the age range of 35-70 years participated in the study after their clinical and biochemical investigations were completed. The liver cirrhosis was diagnosed after performing clinical, biochemical, and abdominal ultrasound (US) investigations.

3. Hepatocellular Carcinoma group (G3):

Thirty HCC patients (18 males and 12 females) with the age range of 35-70 years participated in the study after being diagnosed as having HCC by percutaneous biopsy or radiologically by abdominal ultrasound and Computerized Tomography (CT) according to the guidelines of the American-Association for the Study of Liver Diseases.

The study protocol satisfied the ethical guidelines of the Declaration of Helsinki (1975). Written informed consent was obtained from the patients after informing them with the aim of the investigation and their data were dealt with confidentiality.

Full clinical examination with history taking and investigations were performed for all the study participants. The investigations included: complete blood count (CBC), coagulation profile (INR and Prothrombin time), kidney function tests (Serum BUN and creatinine), liver function tests (bilirubin, AST, ALT, serum albumin), hepatitis markers (HBs Ag and HCV antibody), and serum level of AFP. The specific laboratory investigation included serum p53 and 5-mc%. Radiological tests included abdominal ultrasound to determine the presence of cirrhosis and portal hypertension and exclude the presence of focal hepatic masses and triphasic CT scan for suspected patients with HCC (patients with focal lesion).

Enzyme Immunometric Assay (EIA) kit (Abcam® Assay Designs) was used for quantifying the global DNA methylation status. A double antibody sandwich ELISA kit was used according to the manufacturer's instructions. Enzyme Immunometric Assay kit (Assay Designs' p53 TiterZyme®), a complete kit for the quantifying the mutant and wild-type p53 in humans, was used according to the manufacturer's instructions.

The sample size was calculated using the MedCalc software version 19.0.7 (MedCalc Software) by 80% specifications and an alpha of 0.05 to compare Area Under the Curve (AUC) of AFP (0.7), with the hypothesis that serum global DNA methylation status and P53 could have an AUC of 0.9. At least 30 for HCC and 60 for control (healthy and cirrhotic) were required.

Statistical analysis was done with SPSS software for Windows (version 20). Quantitative data were presented as means $\pm$ standard deviation and their comparisons were performed by ANOVA and post-hoc tests. While qualitative data were presented as frequency distributions with percentage. Nonparametric data of different groups were compared by Chi-square test (X^2). Pearson correlation coefficient (r) was performed for quantitative variables. Evaluation of the diagnosis of each test was assessed by ROC curve analysis and the AUC evaluated the overall test performance. The significance level was set at $P<0.05$.

Results

Fig. 1 and Table 2 show the assay of 5-mC in G1, G2, and G3. The number of patients, range of 5-mC, mean value of each group, and p-values are shown in Table 3. The level of 5-mC in G1 ranged from 0.55 to 1.28% with a mean value of 0.95 ± 0.194%. In G2 patients, the 5-mC ranged from 0.19 to 4.8% with a mean value of 2.24 ± 1.23%, which showed a highly significant increase ($P<0.0001$) compared to the control group (G1). In G3, 5-mC ranged from 0.95 to 16.45% with a mean value of 5.61 ± 4.23%. The significant difference between the mean 5-mC value was observed as comparing G1 and G3 groups ($P<0.0001$).

Table 1: Clinical data in the three studied groups.

	Group I (Control Group).	Group II (Cirrhotic Group)	Group III (HCC Group)
No of Cases	30	30	30
Male	15	18	18
Female	15	12	12
Hb (gm/Dl)	13.8±0.9	12.5±1.5	11.83±1.2
TLC (X10^3/Mm^3)	7.6±2.3	8.3±6.1	5.4±2.7
Platelet (X10^3/Mm^3)	261.7±79.9	132.7±47.9	110.3±86.22
INR	1.1	1.1	1.7
Hbs-Ag	0.00	14 (46.66%)	10 (33.33%)
Hcv-Ab.	0	16 (53.33%)	20 (66.66%)
γ-GT	32.7±12.05	144.4±25.10	167.5±28.19
Alb	4.5±0.54	3.5±0.19	3.1±0.18
Alk-phos	98.3±16.58	240.9±30.27	337.0±87.25
Bili (T)	0.8±0.10	2.2±0.43	2.3±0.45
Bil (D)	0.2±0.04	1.2±0.18	1.2±0.23
SGPT	21.3±6.51	123.8±14.57	168.4±20.71
SGOT	19.2±5.53	124.1±28.74	151.1±27.74
T.p	7.0±0.54	5.2±0.35	5.0±0.35

Table 2: Statistical analysis of 5-mC percentage in control and different tested groups.

	G1	G2	G3
No. of cases	30	30	30
Range	0.73	4.69	15.49
Mean 5-mC	0.95	2.24	5.61
Standard Deviation	0.19	1.23	4.23
Standard Error	0.04	0.23	0.77
Mean ± SD	0.95±0.19	2.24±1.23	5.61±4.23
G1	-	<0.0001	<0.0001
G2	<0.0001	-	<0.0001
G3	<0.0001	<0.0001	-
ANOVA	P<0.0001		

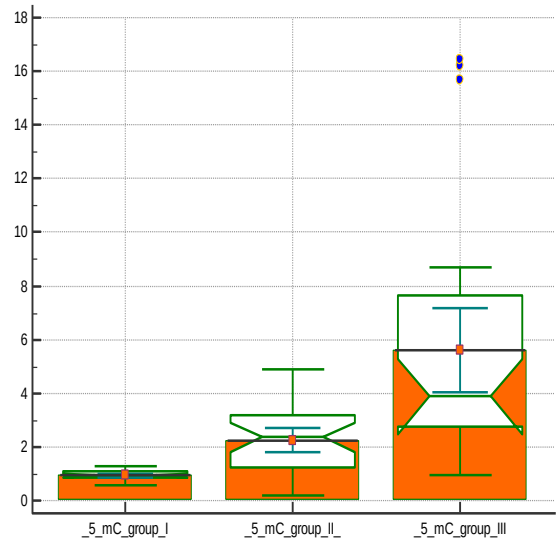

Figure 1: Mean levels ± SD of 5-methylcytosine in control and different tested groups.

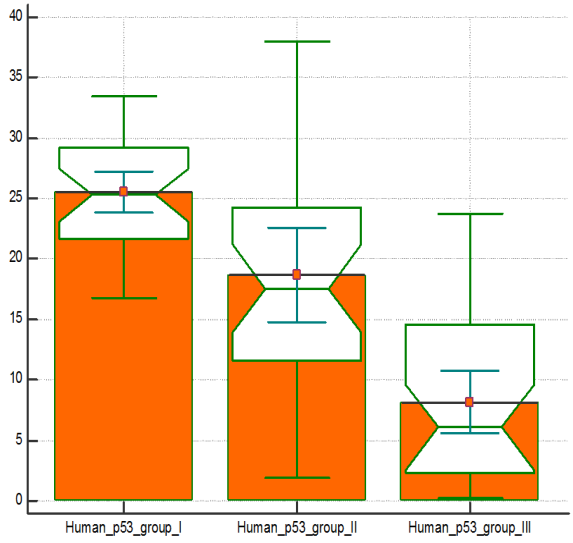

Figure 2: Mean levels ± SD of P53 pg/ml in control and different tested groups.

The assay of P53 in GI, G2, and G3 groups are shown in Table 4 and Fig. 2. The number of patients, Range of P53 pg/ml, the mean value of each group, and p-values are shown in Table 4. The level of P53 in G1 ranged from 16.7 to 33.48 pg/ml with a mean value of 25.51 ± 4.5 pg/ml. In G2, the P53 pg/ml ranged from 16.72 to 33.48 pg/ml with a mean value of 18.68 ± 10.42 pg/ml, which showed a highly significant increase (P<0.0001) as compared to G1. In G3, P53 ranged from 0.16 to 23.78 pg/ml with a mean value of 8.17± 6.8 pg/ml. The significant differences between the mean P53 value was observed as comparing G1 and G3 groups (P<0.0001).

AUC of P53 was 0.894 at cutoff ≤14.77 pg/ml, with a sensitivity of 83.33%, the specificity of 85%, positive predictive value of 73.16, and negative predictive value of 91.03 in diagnosing the hepatocellular carcinoma.

Table 3: Statistical analysis of P53 values in control and different tested groups.

	G1	G2	G3
No of cases	30	30	30
Range (pg/ml)	16.7	36.0	23.6
Mean P53	25.51	18.68	8.17
Standard Deviation	4.5	10.42	6.81
Standard Error	0.83	1.9	1.24
Mean ± SD	25.51±4.5	18.68±10.42	8.17±6.81
G1	-	<0.0001	<0.0001
G2	<0.0001	-	<0.0001

The results of the assay of AFP in GI, G2, and G3 are shown in Table 5 and Fig. 3. The number of patients, mean values of each group, and p-values are summarized in Table 7. The mean value of AFP in G1 was 5.22 ± 2.39 ng/ml. In G2, the mean value of AFP was 21.28 ± 27.16 ng/ml, which showed a highly significant increase (P<0.0001) as compared to G1. In G3, the mean value of AFP was 262.4 ± 431, which showed a highly significant increase (P<0.0001) as compared to G1.

AFP cutoff was 35 ng/ml, with a sensitivity of 65%, the specificity of 95%, the positive predictive value of 87%, and a negative predictive value of 84% in the diagnosis of hepatocellular carcinoma.

Table 4: Statistical analysis of Alpha-Fetoprotein (AFP) in control and different tested groups.

	G1	G2	G3
No of cases	30	30	30
Mean AFP (ng/ml)	5.22	21.28	262
SD	2.39	27.16	431
Mean ± SD	5.22±2.39	21.28±27.16	262±431
G1	--	<0.001	<0.001
G2	<0.001	--	<0.001
G3	<0.001	<0.001	--
ANOVA	P<0.001		

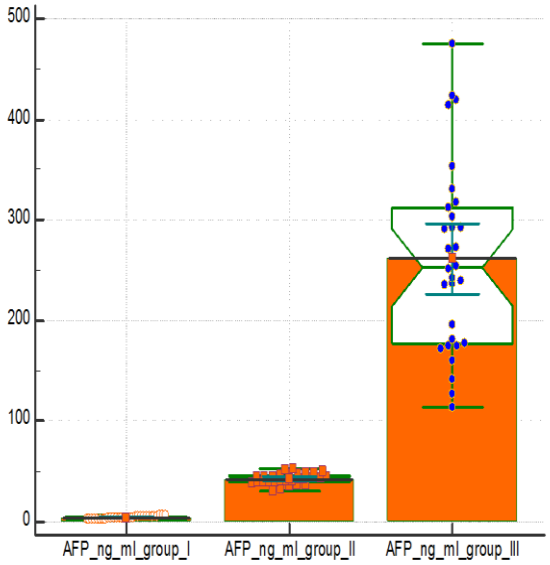

Figure 3: Mean levels ± SD of Alpha-Fetoprotein (pg/ml) in control and different tested groups.

Table 5: Diagnostic performance of serum AFP and serum 5-mC% and p53 in tested groups.

	5-methylcytosine (5-mC)	tumor suppressor protein p53	Alpha-Fetoprotein (AFP)
Associated criterion	>1.3	≤14.77	>35
Sensitivity	96.67	83.33	65
Specificity	65	85	95
positive predictive value	57.46	73.16	87
negative predictive value	97.06	91.03	84

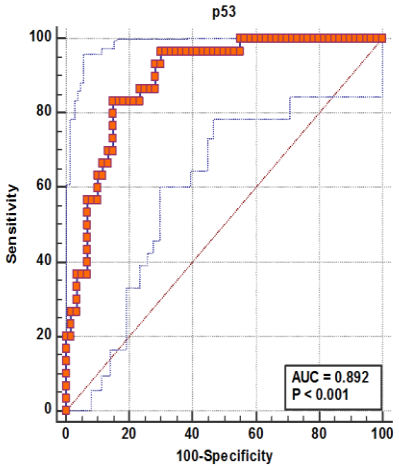

Figure 4: ROC curve diagnostic performance of P53 in tested groups.

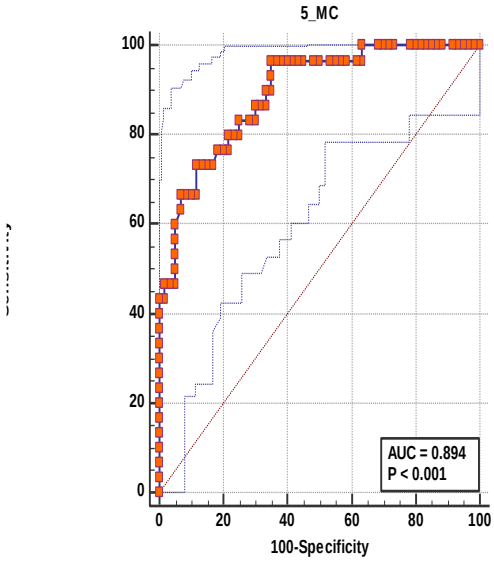

Figure 5: ROC curve diagnostic performance of 5-mC in tested groups.

Discussion

HCC was mostly diagnosed by AFP as the only serological available marker. However, the sensitivity of this marker is still questionable (Zhou et al., 2006). Therefore, the availability of another sensitive serological marker to differentiate hepatocellular carcinoma and benign liver lesions would be very valuable for the early and specific diagnosis of HCC (Wu et al., 2006).

In the current study, AFP level was significantly higher among patients of G3 than those of G1 ($p<0.002$) and G2 ($p< 0.003$). However, the difference between G2 and G1 was insignificant ($p<0.8$), which is in agreement with Wu and colleagues (Wu et al., 2006). Zhu and colleagues (Zhu et al., 2013) showed that serum AFP was significantly higher in the G3 when compared to G1 or G2. They reported that the increase in the selective transcriptional activation in the AFP gene in the malignant hepatocytes results in the increased secretion of AFP during the development of HCC to inhibit the immune response of liver cancer cells.

Sherman (Sherman 2001) verified the AFP cutoff value at 20 ng/ml for diagnosing HCC with 51.9% sensitivity and 86.3% specificity. Consequently, he considered that AFP is an insufficient screening test due to its limited capacity for early detection of new cases when compared with the use of imaging techniques.

AFP concentration sat value of 20 ng/ml with low specificity but fair sensitivity (60%); that is, AFP surveillance would loss 40%, whereas at higher cutoffs of 200 ng/ml the sensitivity decreases to 22% with elevated specificity. Therefore, the cutoff decrease means that extra HCCs would be identified but at the cost of a progressive increased false-positive rate. Sherman (Sherman 2001) stated that an important limitation of using AFP for HCC surveillance is the AFP-negative HCC rate. Up to 50% of minor

HCCs, even with bigger lesions, don't secrete AFP and in 20% of them, there is no increased levels. Regarding the AFP, the cutoff was 35 ng/ml, with the sensitivity of 65%, specificity of 95%, positive predictive value of 87%, and negative predictive value of 84% in diagnosing HCC.

Regarding 5-mC and P53, there was a significant difference between G3 and G2, G3 and G1, as well as G2 and G1. AUC of 5-mC was 0.894, at cutoff >1.3%, with a sensitivity of 96.67%, specificity 65%, the positive predictive value of 57.46%, and negative predictive value of 97.06% in in diagnosing HCC.

The significant difference in the mean value of P53 was observed as comparing G1 and G3 groups (P <0.0001). AUC of P53 was 0.894 at a cutoff of ≤14.77 pg/ml, with a sensitivity of 83.33%, specificity of 85%, positive predictive value of 73.16, and negative predictive value 91.03 in diagnosing HCC. So, when we combine both of them, the sensitivity will increase.

Conclusion

Combining the serum 5-mC with P53 can serve as a new biomarker for the diagnosis of HCC with a higher sensitivity than AFP.

Consent for Publication

Written informed consents were obtained from the participants after informing them with the purpose of the study and their data were dealt with confidentiality.

Accessibility of Data and Materials

Not applicable.

Funding

None.

Conflict of Interests

The authors declare no conflict of interest.

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Declared none.

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