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RESEARCH ARTICLE

CRISPR KNOCKOUT OF TAX1BP3 REDUCES EXPRESSION OF P53 IN HUMAN PAPILLOMA NEGATIVE AND HUMAN PAPILLOMA POSITIVE CERVICAL CARCINOMA CELLS.

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Abstract

Background: The PDZ protein Tax 1 binding protein 3 (Tax1BP3) is unique since, unlike other PDZ proteins, it contains a single rather than multiple PDZ domains. Previous work has shown Tax1BP3 is a target of oncoproteins from two separate human tumor viruses and it is known to regulate Rho and wnt/ β -catenin signaling. Most importantly Tax1BP3 has been shown to promote degradation of the p53 protein in glioma cells although it is not clear whether it performs this same function in tumor virus positive cell types .

Methods: The cervical cancer cell lines C33A (HPV negative), C33AE6 (HPV16 E6 transfected) and CaSki (HPV16 positive) were stably transfected with a CRISPR/Cas9 Tax1BP3 knockout construct. Levels of Tax1BP3 mRNA were initially determined in puromycin resistant monoclonal cells by RT-PCR. Tax1BP3 and p53 protein levels were then analyzed by a combination of western immunoblotting and flow cytometry.

Results: Both bi and monoallelic Tax1BP3 targeted cell lines were isolated for all three cell types which showed either partial or complete silencing of Tax1BP3 expression. Irrespective of cell type, all cells which had reduced or absent Tax1BP3 expression showed much lower levels of p53 protein than control cells..

Conclusion: Unlike previous findings with glioma cells, the presented data showed that reduced levels of Tax1BP3 directly correlated with reduced levels of p53 protein expression. Furthermore, this effect was seen in cells whether or not they expressed mutant (C33A) or wild type (CaSki) p53 or full or partial HPV genome (CaSki and C33AE6). These results suggest that Tax1BP3 regulates p53 protein levels in HPV positive and negative cells by a novel and non-canonical means.

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Abbreviation:-

Tax1BP3: Tax 1binding protein 3

CRISPR/Cas9: Clustered Regularly Interspaced Short Palindromic Repeats/CRISPR associated nuclease 9

HPV16: human papilloma virus type 16

Introduction:-

Tax1BP3 (Tax 1 binding protein 3), also known as Tax interacting protein 1 (Tip-1) or glutaminase interacting protein (GIP) is a multifunctional protein targeting many proteins in diverse signaling pathways involved in cancer and other disease (Mohanty et al., 2015). Tax1BP3 consists of single PDZ domain which makes Tax1BP3 unique since other PDZ proteins possess multiple PDZ domains (Wang et al., 2014).

It has been previously shown that Tax1BP3 interacts with the human T-cell leukemia virus type 1 Tax (Rousset et al., 1998), and HPV16 E6 oncoproteins (Hampson et al., 2004). Most significantly, unlike other E6 protein targets (Ainsworth et al., 2008), Tax1BP3 was not degraded by its interaction with E6 which led to speculation that this interaction may produce an oncogenic gain-of-function (Hampson et al., 2004). In this regard Tax1BP3 has also been shown to interact with the guanidine exchange factor ARHGEF16 which resulted in E6-dependent activation of the Rho protein cdc42 in cervical carcinoma cells which is known to promote malignant transformation and progression (Oliver et al., 2011). Tax1BP3 is also known to regulate both Rho-A (Hampson et al., 2004) and β -catenin/wnt signaling (Kanamori et al., 2003) which are both considered as crucial pathways involved in neoplastic transformation. Elevated Tax1BP3 was also detected in invasive breast cancer combined with xenograft studies which demonstrated its ability to regulate tumor cell motility and adhesion (Han et al., 2012).

Other work has shown that Tax1BP3 may play a role in the development of brain tumors. For example it is known that it interacts with the DTX1 (Deltax ubiquitin E3 ligase 1), STAU1 (staufen double stranded RNA binding protein 1), glutaminase L and BAL2 (2,3 dimercaptophenol) proteins which are involved in normal brain function and yet abnormalities in their function have been linked to the formation of brain tumors (Zencir et al., 2013). Moreover, Tax1BP3 has also been shown to interact with the guanidine exchange factor ARHGEF7 and to regulate Rho signaling in glioblastoma cells and anti Tax1BP3 antibodies have been shown to control tumor growth in tumor xenografts (Wang et al., 2014, Yan et al., 2016). Furthermore, most relevant to the current study, Tax1BP3 has been shown to promote the ubiquitination and degradation of the tumor suppressor p53 in glioma cell lines (Han et al., 2012). CRISPR/Cas 9 (Clustered Regularly Interspaced Short Palindromic Repeats/CRISPR associated nuclease 9) is RNA-guided genome editing tool in which 20 nt guide RNA sequence direct the cas9 to the target gene by base pairing, resulting in site specific DNA double strand breaks (DSBs) which subsequently repaired by non homologous end joining (NHEJ) pathway or homology directed repair (HDR) pathway result in disruption of the target gene (Ebina et al., 2013, Cong et al., 2013).

CRISPR/Cas 9 system is efficient, easy to design and simple to use in comparison to other programmable nucleases such as zinc finger nuclease (ZFNs) and transcription activator like effector nucleases (TALENs) which are difficult and time consuming to design (Gaj et al., 2013, Mali et al., 2013).

With these advantages CRISPR/Cas 9 system has become an efficient and convenient tool for genome editing and has been used to study many cancer associated genomic mutations (Zhen et al., 2014, Lin et al., 2014).

In light of this work, we now present a study on the effects of CRISPR mediated gene knock out of Tax1BP3 on p53 levels in cultured HPV positive and negative cervical carcinoma cell lines expressing wild type and mutant p53 in the presence of the full or partial HPV16 genome.

Material and methods:-***Cell lines and Cell culture condition***

Three cervical cancer cell lines C33A (HPV negative), C33AE6 (HPV16 E6 transfected) and CaSki (HPV16 positive) were grown in Roswell Park Memorial Institute medium (RPMI) -1640 (Invitrogen Ltd.) supplemented with 10% fetal bovine serum (FBS) and 2 mM L-glutamine (Sigma-Aldrich).

CRISPR/Cas9 construct , stable gene transfection and Tax1BP3 silencing

Genome-Wide knockout kit using CRISPR /Cas9 gene knockout/ knock in kit specific for Tax1BP3 was obtained from (Origene) was used the kit used to knockout Tax1BP3 gene through gene specific guide vector and knock in a functional cassette containing GFP (green fluorescent protein) gene and puromycin resistant gene by using a donor vector . Tax1BP3 Single guide RNA (sgRNA) was cloned into pCas guide vector and were designed to target the amino-terminal region of the Tax1BP3 open reading frame (ORFs)Tax1BP3sgRNA Sequence: CGTTTCCCGCTCTGCGAGG.

A negative scramble gRNA control cloned into pCas guide vector was also provided . All cell lines were stably transfected with guide and donor vector provided with knockout/knock (origene) according to manufacturer's instruction kit and lipofectamine 2000 (Invitrogen) was used as a transfection reagent .Using the identical procedure and culture condition all cell lines were stably transfected with scramble control and donor vector. Then all cells were subcultured for 7 times before monoclonal selection with puromycin the optimal puromycin concentration was (1 µg/ml) for c33A and C33AE6 while (0.2 µg/ml) for caski cell line .

RNA extraction and reverse transcription polymerase chain reaction (RT-PCR)

Total RNA was extracted using Ambion kit (Invitrogen). 1 µg of total RNA were subjected to a reverse transcription (RT) using TetrocDNA Synthesis Kit (Bioline) as recommended by the manufacturer. 1 µl of RT reaction mixture were subjected to PCR. For Tax1BP3 mRNA amplification, the forward primer 5' - GTACTCTGCTGCCGCTTCT-3' and reverse primer, 5'-TCACCAAGCGCTCGGAGGAG-3' were used with thermal cycles set at the condition as follow; 94°C for 2 min , 35 cycles of 94°C 30 sec, 55°C 30sec , 72°C 30sec, followed by 72 °C for 5 min.

western blot analysis and antibodies

western blot experiments were carried out according to standard protocols and were developed by using the enhanced chemiluminescence detection system (GE Healthcare Lifesciences, Buckinghamshire, UK). the following primary antibodies were used The TAX1BP3 mouse monoclonal primary antibody (Abgent) ,the mouse monoclonal primary antibody p53 ab-6 (calbiochem), loading control were mouse monoclonal to GAPDH ab9484 (Abcam). The secondary antibody was polyclonal rabbit antimouse HRP conjugated for western applications(DAKO) .

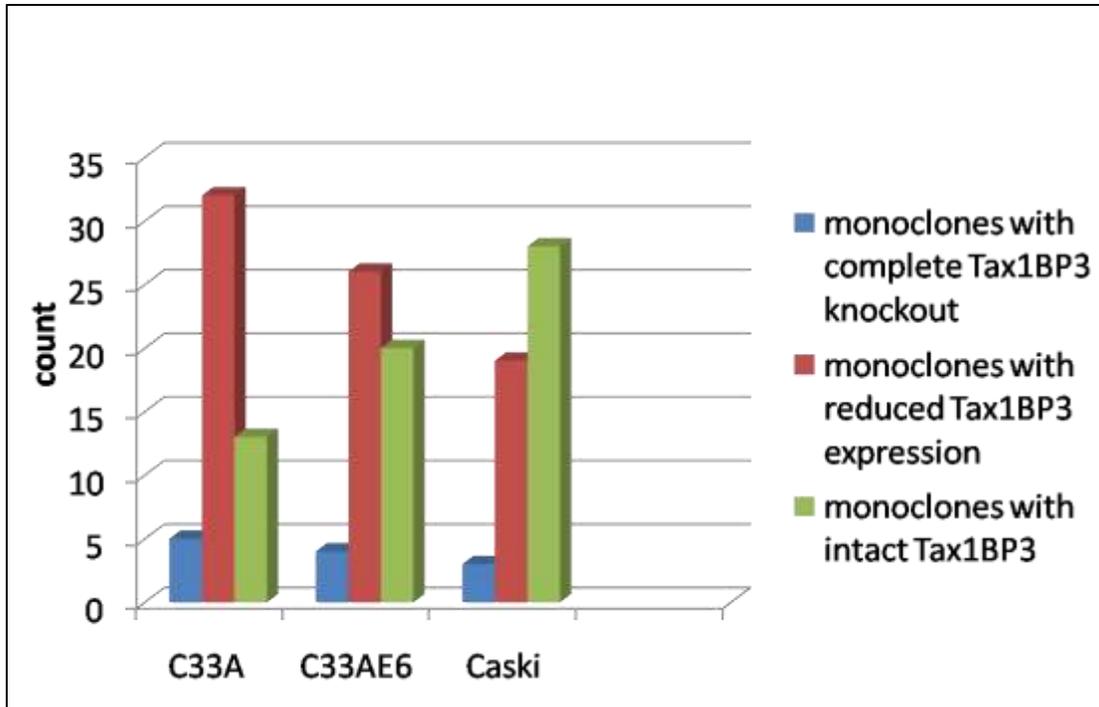
Flow cytometric analyses

Cells were grown in T-25 flasks (Corning, US) to a confluence of 70 % . Cells were harvested and counted , 1 x 10⁶ cells were centrifuged then resuspended in 5 ml of 1 x PBS at 4 °C and washed by centrifugation 5 minutes. The cell pellet was then fixed with 1 ml of ice-cold 70 % Ethanol and stored overnight at 4 °C. The next day, cells were centrifuged and the supernatant was aspirated and the anti p53 antibody diluted 1:1000 (Promega Ltd UK) was added to each sample at 10 mg/ml. The samples were then stored for 1 hour at 4 °C. After this incubation period, samples were centrifuged for 5 minutes at 4 °C, washed with ice-cold 1 x PBS, then centrifuged again for 5 minutes at 4 °C. The 1 x PBS was aspirated until only 300 µl remained over each pellet. These were then resuspended and transferred to a Falcon flow cytometry tube (Jencons Ltd UK). Samples were analysed on a BD FACSCanto™ Flow Cytometer.

Results:-**CRISPR/Cas9-Mediated Targeted Disruption of Tax1BP3 gene**

After stable transfection cells were subjected to puromycin selection for 2 weeks until monoclonal cells with stably integrated plasmid were appeared then we have isolated stable monoclonal cells totally 50 monoclonal cells from each cell line.

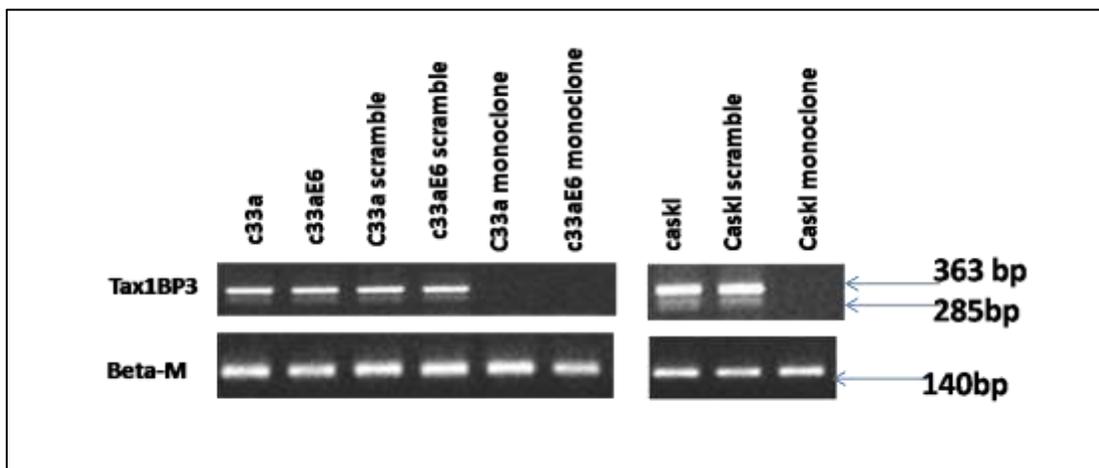
The presented result show that CRISPR/Cas can efficiently inactivate Tax1BP3 in HPV positive and negative cervical cancer monoclonal cells and we were successfully able to isolate completely silenced Tax1BP3 (biallelic knockout) monoclonal cells from all cell lines in addition to many monoclonal cells with partial Tax1BP3 knockout (monoallelic knockout).



(Fig 1) statistical analysis Tax1BP3 expression of 50 stably transfected monoclonal isolated after CRISPR transfection of C33A, C33AE6 and caski cell lines using Pearson's Chi-square or fisher exacttest. The number of monoclonal with affected Tax1BP3 relative to those with intact Tax1BP3 was with high significance <0.001.

Tax1BP3 disruption led to absence of Tax1BP3 mRNA and protein

RT-PCR results confirm absence of Tax1BP3 after CRISPR/Cas application After antibiotic selection RNA were extracted from c33a and c33aE6 monocoles with stably integrated plasmid then RT-PCR was done to analyze Tax1BP3 RNA expression in stable monoclonal. The presented results show the ability of CRISPR/CAS9 perturb Tax1BP3 gene and consequence absence of Tax1BP3RNA in RT-PCR analysis (figure 2).



(Fig 2) RT-PCR analysis showing absence of Tax1BP3 RNA After CRISPR/Cas mediated Tax1BP3 knockout.

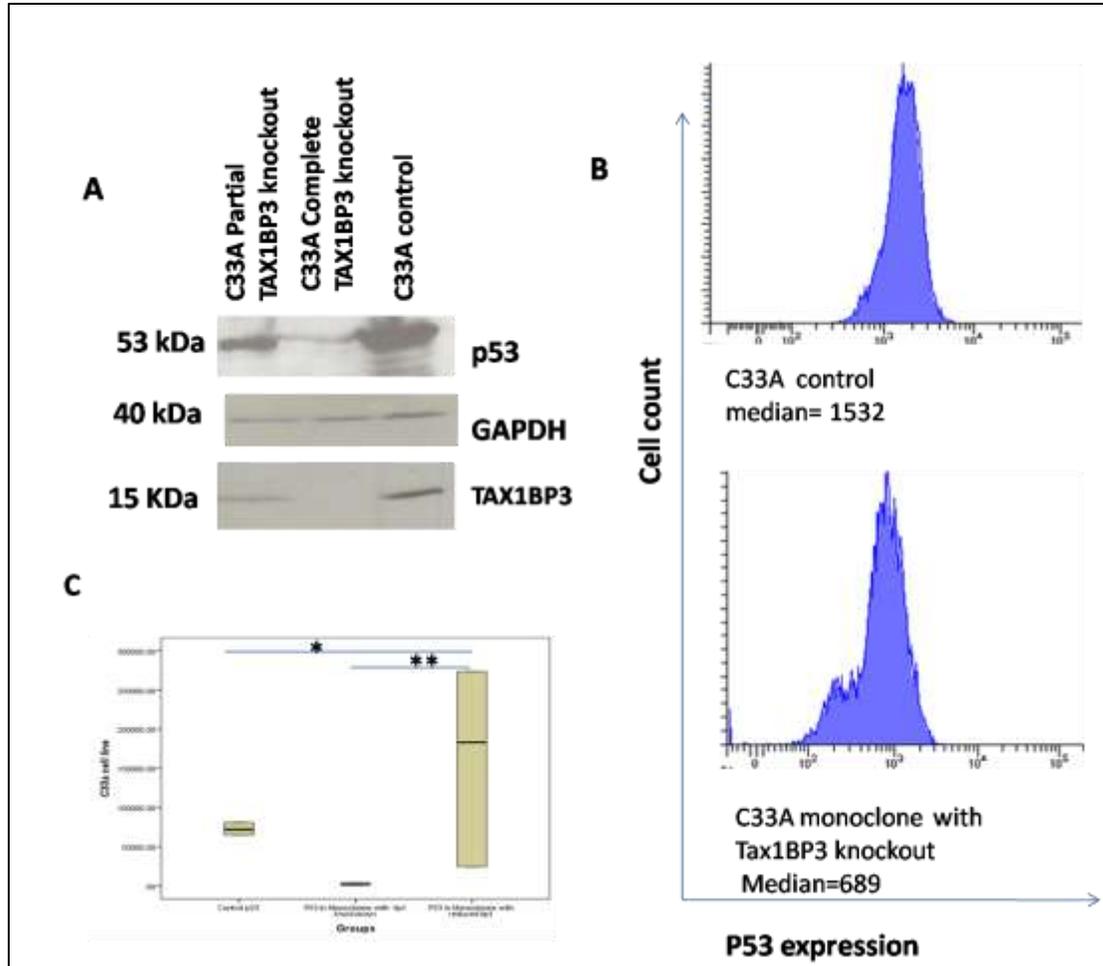
Western blot confirms the effect of CRISPR/Cas system on Tax1BP3 protein level protein were extracted from c33a and c33aE6 and caski stable monoclonal then western blot analysis was done to investigate Tax1BP3 expression. monoclonal with complete knockout show complete absence of protein signal while those monoclonal with partial knockout show reduced expression signals (figure 3,4,5).

Tax1BP3 knockout markedly reduce p53 expression level in HPV positive and HPV negative monoclones

Protein lysate was extracted from C33A, C33aE6 and caski monoclones with complete Tax1BP3 knockout to assess p53 expression level using western blot and interestingly p53 expression levels were markedly reduced in comparison to their controls, taking into consideration that caski cells contain HPV whole genome and express wild type p53 while C33A is HPV negative and express mutant type p53.

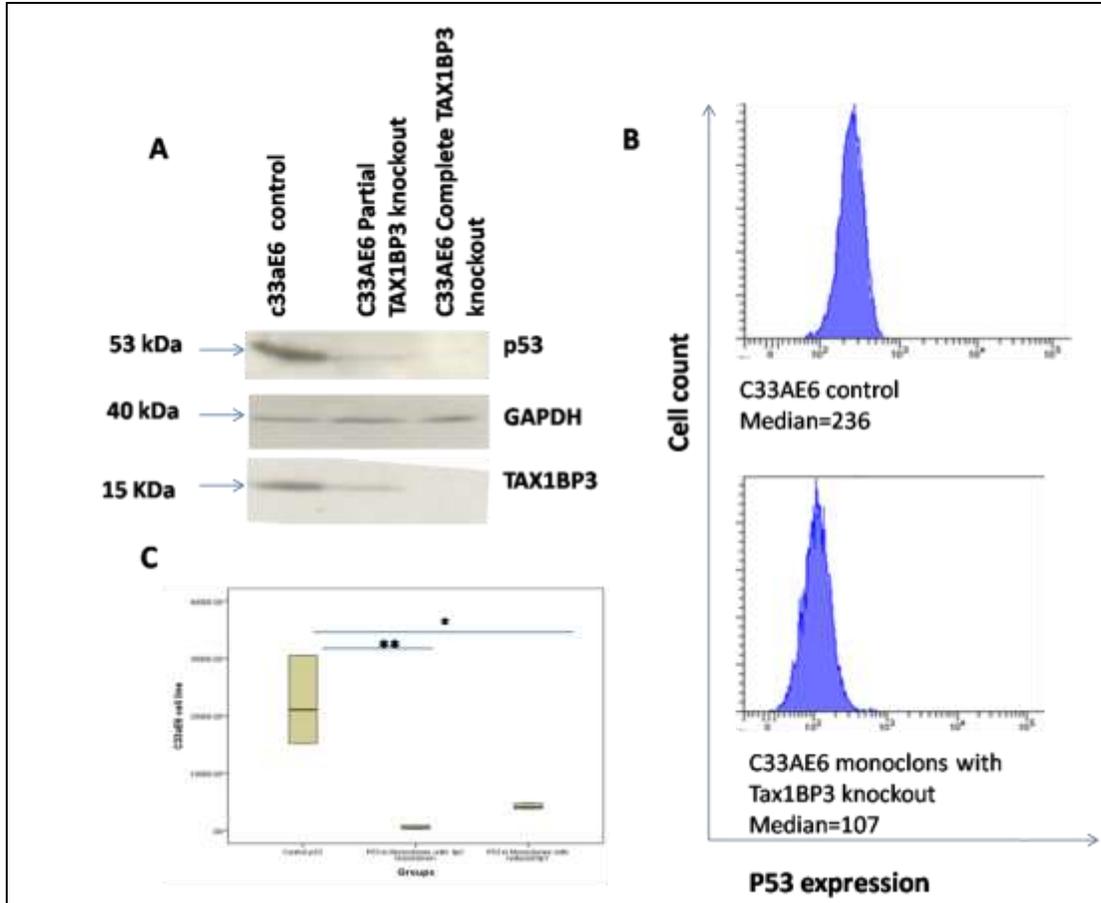
C33AE6 cells are modified C33A cells obtained by stable transfection of the parental C33A cell line with HPV16 E6 cDNA so the cells express only HPV E6 oncogene (partial HPV genome) and contain mutant type p53 the same as C33A cell lines (*Hampson et al., 2001*).

Reduction of p53 expression after Tax1BP3 knockout was also confirmed by flow cytometry showing the same results in all cell lines monoclones (figure 3,4,5).



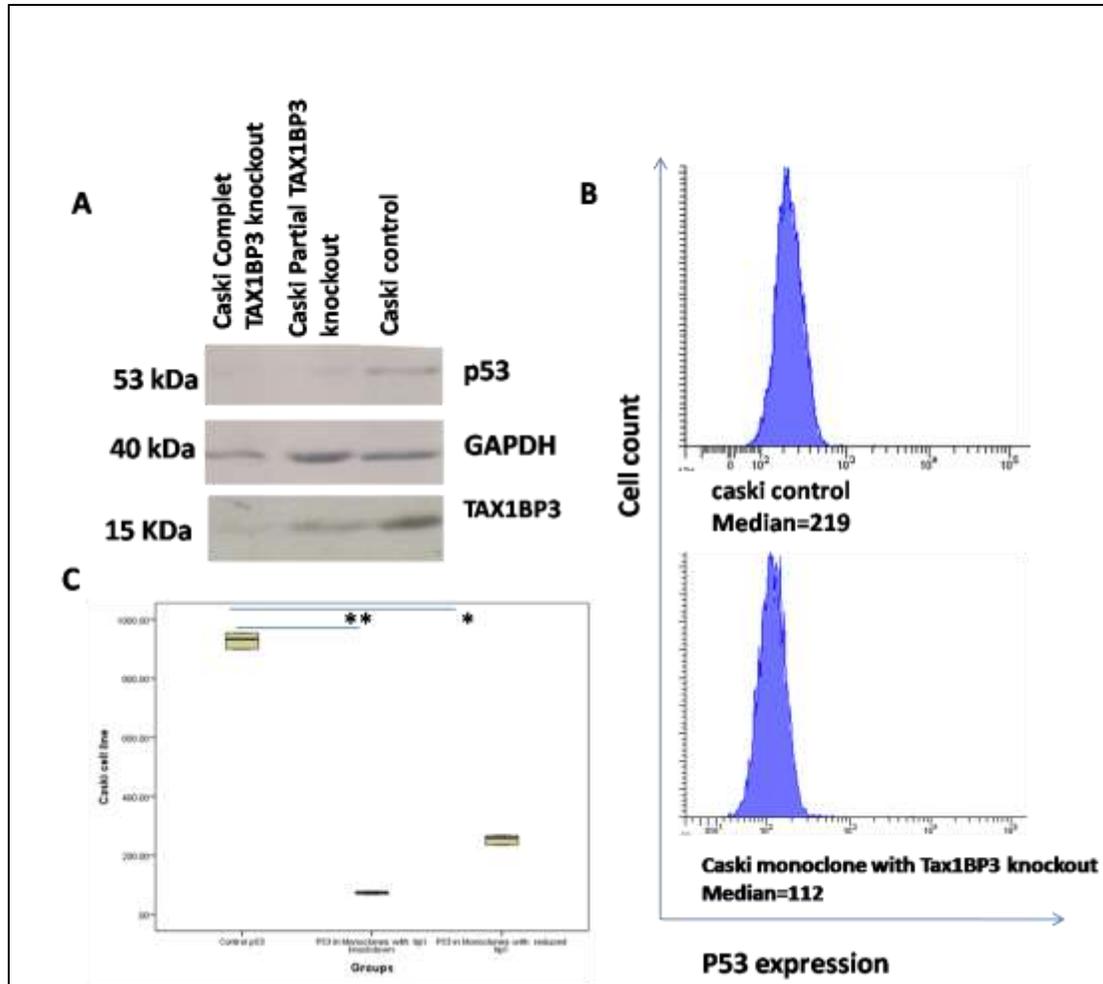
(Fig 3) A) western blot analysis of Tax1BP3 expression of protein extracted from C33A stably transfected monoclones showing the ability of CRISPR/Cas9 to knockout Tax1BP3, both monoclones with complete Tax1BP3 knockout (biallelic) and partial Tax1BP3 knockout (monoallelic) associated with a reduction of p53 expression.

B) Flow cytometric analysis of p53 expression level showing p53 expression reduction in monoclones with CRISPR/Cas mediated Tax1BP3 knockout in comparison to their control. C) Statistical analysis of p53 expression reduction in C33A monoclones using Kruskal Wallis followed by pairwise comparisons (n=6), monoclones with partial Tax1BP3 Knockout show * significance <0.05 relative to Control p53 monoclones with complete Tax1BP3 Knockout show ** high significance <0.001 relative to Control p53.



(Fig 4) A) western blot analysis of Tax1BP3 expression of protein extracted from C33AE6 stably transfected monoclonal cells showing the ability of CRISPR/Cas9 to knockout Tax1BP3, both monoclonal cells with complete Tax1BP3 knockout (biallelic) and partial Tax1BP3 knockout (monoallelic) associated with a reduction of p53 expression.

B) Flow cytometric analysis of p53 expression level showing p53 expression reduction in monoclonal cells with CRISPR/Cas mediated Tax1BP3 knockout in comparison to their control. C) Statistical analysis of p53 expression reduction in C33AE6 monoclonal cells using Kruskal Wallis followed by pairwise comparisons (n=6), monoclonal cells with partial Tax1BP3 Knockout show * significance <0.05 relative to Control p53 monoclonal cells with complete Tax1BP3 Knockout show ** high significance <0.001 relative to Control p53.



(Fig 5) A) western blot analysis of Tax1BP3 expression of protein extracted from caski stably transfected monoclonal showing the ability of CRISPR/Cas9 to knockout Tax1BP3, both monoclonal with complete Tax1BP3 knockout (biallelic) and partial Tax1BP3 knockout (monoallelic) associated with a reduction of p53 expression.

B) Flow cytometric analysis of p53 expression level showing p53 expression reduction in monoclonal with CRISPR/Cas mediated Tax1BP3 knockout in comparison to their control. C) Statistical analysis of p53 expression reduction in caski monoclonal using Kruskal Wallis followed by pairwise comparisons ($n=6$), monoclonal with partial Tax1BP3 Knockout show * significance <0.05 relative to Control p53 monoclonal with complete Tax1BP3 Knockout show ** high significance <0.001 relative to Control p53.

Discussion:-

The work presented describes the ability of the bacterial RNA guided endonuclease of CRISPR/Cas9 system to efficiently inactivate Tax1BP3, many previous studies have reported the technology of siRNA or shRNA to knockdown Tax1BP3 (Oliver et al., 2011, Han et al., 2012) however their effect is temporary, counteracted by human cells in long run and cannot be inherited narrowing its application especially in therapeutic purposes (Hu et al., 2014, Xiao-Jie et al., 2015)

Monoclonal with reduced Tax1BP3 expression were also obtained which probably result from a monoallelic knockout in this regard the paired-gRNA guided double nicking Cas9 system could be better applied in the future to increase cas9 system specificity and decreases potential off targets (Ran et al., 2013).

The experiment was extended to analyze the prospective consequence of Tax1BP3 knockout on the expression level of p53 in HPV positive (c33aE6 and caski) and HPV negative cell lines and interestingly after

Tax1BP3 knockdown a significant reduction in p53 expression was detected in all cell lines monoclonal taking into consideration that both c33a and c33aE6 contain mutant type p53 while caski cell line contain wild type p53. Reduced Tax1BP3 expression (partial knockout) was also associated with a reduction in p53 expression in all monoclonal lines.

Tax1BP3 interacts with many intracellular protein partners such as Rho GTPase (Reynaud et al., 2000), glutaminase L (Olalla et al., 2001), potassium channel Kir2.3 (Alewine et al., 2006), β catenin (Kato et al., 2007), HPV16 E6 (Hampson et al., 2004), Rho guanine exchange factor 16 (Oliver et al., 2011), plexinD1 and AT hook DNA binding containing 1 (AHDC1) proteins (Shalaby et al., 2011). Of particular interest to note that Tax1BP3 was documented to inhibit the proliferation of colon cancer cells suggesting that Tax1BP3 might function as a tumor suppressor and on the other hand, elevated Tax1BP3 expression levels were detected in human invasive breast cancer (Han et al., 2012) and human glioblastoma and was associated with advanced stages and poor prognosis revealed an oncogenic function of Tax1BP3 (Wang et al., 2014).

What is the relationship between p53 and Tax1BP3?

p53 normally expressed in low level due to its interaction with Murine Double Minute 2 (MDM2) which act as E3 ubiquitin ligase promoting the proteasome mediated p53 protein degradation (Gu et al., 2006). p53 is also regulated by ARF which activates p53 by inhibiting MDM2 mediated p53 degradation. In a previous study on glioma cells, Tax1BP3 was shown to enhance the protein interaction between LZAP and ARF leading to polyubiquitination and degradation of p53 (Han et al., 2012).

However Opposite to those observations another study on osteosarcoma cell line demonstrated that interaction between LZAP and ARF leading to maintaining p53 stability and increased p53 transcriptional activity (Wang et al., 2006). The mechanism by which Tax1BP3 affect the expression of p53 is not completely understood. Taking together, we speculate that Tax1BP3 might regulate p53 expression through its interaction with LZAP protecting p53 from degradation and might be considered as one explanation for reduced p53 expression after Tax1BP3 knockdown. It is noteworthy that previous work has shown Direct evidence between β -catenin and p53 demonstrating that excess β -catenin can induce an accumulation of active p53 which explain, at least in part, loss of p53 activity in tumors harboring deregulated β -catenin, such as colorectal cancer and hepatocellular carcinoma (Han et al., 2012) taking those together with the fact that Tax1BP3 interact with β -catenin and inhibit its transcriptional activity (Lowy et al., 2006) thus we hypothesize that Tax1BP3 knockdown may be associated with down regulation of β -catenin and consequently p53 expression.

We noted that other report showed that Tax1BP3 knockdown remarkably change the cellular morphology in glioma cells (Wang et al., 2014). However, we did not see any cellular morphology difference in all monoclonal lines after Tax1BP3 knockdown in comparison to the original cell line or the scramble control. This difference might be due to the different cellular context between glioblastoma cell and cervical cancer cells.

As Tax1BP3 interact with several intracellular proteins, we believe that it would be interesting to investigate whether Tax1BP3 knockdown can affect the expression level of any other Tax1BP3 partners especially β -catenin and LZAP and may help to understand the exact mechanism by which Tax1BP3 could affect the expression of p53.

Conclusion:-

The presented data showed that reduced levels of Tax1BP3 directly correlated with reduced levels of p53 protein expression. This effect was seen in cells whether or not they expressed mutant (C33A) or wild type (CaSki) p53 or full or partial HPV genome (CaSki and C33AE6). These results suggest that Tax1BP3 regulates p53 protein levels in HPV positive and negative cells by a novel and non-canonical means.

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