DISCUSSION

In the present study, we demonstrate differential expression of microRNAs miR-21 and let-7a in cervical cancer cell lines and tumor tissues derived from cervical pre-cancer and cancer tissue lesions. The expression of miR-21 was significantly higher while let-7a showed a low level of expression in cervical cancer tissues. However, such a reciprocal kinetics was not observed in tissues of LSIL and HSIL which over-expressed let-7a. While miR-21 showed direct association, let-7a expression was inversely related to STAT3 expression and activation in HPV16-positive cervical cancer lesions. Both, miR-21 and let-7a were found differentially expressed with respect to levels of oncoprotein E6 in HPV-positive lesions. Over-expression of miR-21 was found associated with elevated levels of other STAT3 regulated gene products MMP-2 and MMP-9 and decreased levels of its known target gene products, PTEN and TIMP-3.

STAT3 targeting either by commercially available siRNA or pharmacological agents such as curcumin and statin resulted in decreased STAT3 expression which promoted reduction of miR-21 expression in SiHa cervical cancer cells. miR-21 targeting favors gain of PTEN expression whereas Let-7a over-expression suppressed STAT3 in cervical cancer. HPV16 E6 oncoprotein inhibition showed increase in let-7a expression and reduced miR-21 levels. Aberrant STAT3 expression plays an important role in cervical carcinogenesis. Our current understanding of the role of STAT3 focuses on its ability to deregulate cellular gene expression at the transcription level through its interaction with many cellular factors.

miR-21 increases as a function of severity of lesions

miR-21 showed an increase in expression with progression and severity of the disease in HPV16+ pre-cancer and cancer cases as compared to HPV-negative and normal tissues. Moreover, the degree of miR expression was highly variable within cancer cases in our study that increased maximally in tissues from clinically advanced stage IV lesions. miR-21 has been shown to over-expressed in cervical cancer cell lines (Lui et al. 2007). Aberrant expression of miR-21 in cervical carcinogenesis has been shown to be involved in upregulation of proliferation and down-regulated of apoptotic genes (Lui et al. 2007; Wang, X. et al. 2008; Yao, Q. et al. 2009; Yao, T. and Lin 2012).
Let-7a showed reciprocal correlation with the grades of lesions and STAT3 expression

Contrary to miR-21, let-7a had high expression level in tissues normal cervix, LSIL and HSIL tissues as compared to low level in cancer lesions and it is unclear why let-7a showed high expression in pre-cancer tissues as compared to control. The let-7 is known to represses cell proliferation pathways in human cells (Johnson et al. 2007). Interestingly, let-7 genes locate to loci deleted in multiple types of cancers, such as breast, ovary, urothelium, including cervical cancers (Calin et al. 2004). Similar to our findings for let-7a, other studies have reported let-7a as a downregulated gene in several cancers e.g. burkitt lymphoma, kidney cancer and ovarian cancer (Lu et al. 2007; Sampson et al. 2007; Nam et al. 2008) indicating its tumor suppressive role. However, its role in cervical cancer is not well defined; moreover, its upregulation specifically during pre-cancer is intriguing and needs further investigation.

Like our earlier report (Shukla et al. 2010), present investigation demonstrated aberrantly expressed and constitutively active STAT3 in cervical pre-cancer and cancer lesions. Our immunoblotting assay revealed that aberrant STAT3 activity increases as a function of severity of the disease from pre-cancer to cancer during cervical carcinogenesis. Expression of STAT3 was elevated at transcript level in HSIL and cancer lesions. Over-expression of activated STAT3 was accompanied by elevated levels of miR-21. Like constitutively active STAT3, aberrant expression of miR-21 expression is involved in cervical carcinogenesis (Lui et al. 2007; Wang, X. et al. 2008; Yao, Q. et al. 2009; Yao, T. and Lin 2012). STAT3 has been shown to transcriptional induction of miR-21 through a highly conserved enhancer (Loffler et al. 2007), thus making it one of the most important mediator of STAT3’s downstream effector mechanisms. Our earlier IHC based analysis have shown presence of STAT3 and pSTAT3 particularly in tumor cells in pre-cancer & cancer lesions but being a tissue averaging study a minor contribution from contaminating stromal cells cannot be excluded.

Let-7a physically interacts with STAT3 and regulates its expression

Our results showed high STAT3 transcript level in tumor biopsies specifically where the let-7a expression was expressed at low level. Negative correlation of let-7a
with STAT3 expression has been observed in virally-induced hepatocellular carcinoma (Wang, Y. et al. 2010). In this disease model, viral protein HBx was found to target let-7a that resulted in upregulation of STAT3 in HCC patients. Therefore, it is likely that some of the viral proteins of HPV could be directly involved in targeting the expression of let-7a in cervical cancer cells. These authors showed that let-7a physically interacts with the 3’UTR of STAT3 to negatively regulate its cellular expression. This finding is in agreement to our present observation indicating low level of let-7a could have unchecked expression of STAT3 in cancer lesions. let-7a mediates downstream effects of IL-6 over-expression on STAT3 phosphorylation, when let-7a is over-expressed in cholangiocytes. STAT3 phosphorylation is increased through down-regulated of neurofibromatosis gene-2, NF-2 a tumor suppressor gene and a known modulator of STAT3 activation, and a putative target for let-7a (Meng et al. 2007).

**Association of miRs expression with multiple HPV types**

Although we found majority of the cases in our study were HPV16 positive, other types of HPV in cancer and pre-cancer tissue may affect the expression of miR-21 and Let-7a. We checked multiple HPV typing in our cases and found presence of HPV33 in one of the cancer tissue and HPV55, 56 and M7 types along with HPV16 co-infection in three cancer tissues. We found high expression of miR-21 in HPV55 and 56 infected cancer tissues while HPV33 positive sample showed high let-7a expression. However, in view of low number of samples we could not do further analysis. Leaving apart the co-infection issues, all pre-cancer or cancer tissues found positive for HPV16 that showed high levels of HPV16 E6 were accompanied by relatively high levels of miR-21 but low levels of let-7a. Several recent studies have shed light on the possible interactions between HPV and microRNAs. HPV associated/induced alteration in the expression of miRNAs has been reported in cervical carcinoma cell lines (Martinez et al. 2008). Similar to our observations, there is an inverse correlation between miR-125b and HPV DNA in productive infection in cervix (Nuovo et al. 2010). HPV infection in cancer cells is able to negatively influence miR-34a levels through E6 inhibition of p53 (Wang, S. and Olson 2009). Similarly, HPVE6/E7 can downregulate expression of tumor suppressive miR-218 (Martinez et al. 2008) and miR-203 (Melar-New and Laimins
2010). However, it needs to investigate how HPV oncoproteins regulate these STAT3-related miRs or vice versa.

We observed that cancer tissues related to advanced clinical stages or histologically showing high-grade PDSCC lesions showed comparatively higher levels of miR-21 and reduced level of let-7a expression among different cancer tissues and this trend increased with the severity of the disease, with highest miR-21 but lowest let-7a levels in highly advanced cancer cases suggesting that there could be a direct association of HPV16 oncogene expression in conjunction with aberrantly expressed STAT3 signaling.

Higher levels of miR-21 relates with MMP-2 and MMP-9 over-expression and reduced TIMP-3

In an effort to transcriptional upregulation of miR-21 in cervical cancer cells, our results of two other STAT3-controlled gene products, MMP-2 and MMP-9 revealed an upregulation in cervical cancer cell lines and cancer lesions that corresponded with miR-21 expression. STAT proteins are involved in the modulation of activity for both promoters of MMPs and TIMPs (Gatsios et al. 1996; Korzus et al. 1997; Catterall et al. 2001; Tsareva et al. 2007). miR-21 has been shown to contribute to glioma malignancy by down-regulated of MMP inhibitor, TIMP-3 which leads to activation of MMPs (Gabriely et al. 2008). These observations indicate that STAT3-mediated events include upregulation of MMPs that could be partially mediated through activation of miR-21 that negatively regulates TIMP3. Our immunoblot analysis for TIMP-3 revealed reduced expression in miR-21 over-expressed cancer lesions, possibly this could be an effect of miR-21-induced silencing TIMP-3 transcripts. Reduced expression of TIMP3 has been associated with poor disease-free survival (Mylona et al. 2006). TIMPs contain a putative consensus miR-21 binding site indicating negative modulation of TIMP-3 by miR-21.

Lower levels of PTEN are accompanied by increased miR-21 levels

In addition to TIMP-3, PTEN which is another molecular target of miR-21 was also found specifically down-regulated in miR-21 over-expressing lesions. A recent study showed that miR-21 targets PTEN gene through a binding site on 3’-UTR in hepatocellular carcinoma (Meng et al. 2007). Low levels of PTEN have
been reported in cervical cancer (El-Mansi and Williams 2006; Lee et al. 2006; Zhang et al. 2007) thus indicating a potential role of miR-21 in targeting the negative regulator which may further promotes aberrantly activated STAT3. Although we initiated our study to investigate miR-21 as downstream target of STAT3 & explored the expression of its target protein PTEN, we observed a significantly low level of PTEN in cervical biopsies that expressed high levels of miR-21. Low levels of PTEN, in general, have been reported in cervical cancer lesions. Earlier studies showed that among other negative regulators of STAT3, PTEN is responsible for its dephosphorylation and controls overall levels of transcriptionally active phospho (Y705) form of STAT3 (Sun and Steinberg 2002) and thus miR-21 may play an indirect role in promoting phospho-STAT3 levels amplification of STAT3 levels observed in cervical carcinogenesis.

Although, HPV16/18 infected cervical cells have over-expression of viral proteinsE6 and E7, which repress/degrade tumor suppressor proteins p53 and Rb to inflict global disturbance on cellular signaling network and molecular expression profile. A "cherry-picking" of microRNAs such as miR-21 may not be specific and important enough to serve as a major set of biomarkers for diagnosis of cervical cancers. However, it is important to understand that because of redundancy in their regulation and pleiotropic effects induced by having multiple targets independent prognostic/diagnostic value of miR-21 and let-7a could be low, however in contrast with oncogenic transcription factors that act as molecular/cellular switches and control (e.g. miR-21) or get control (e.g. let-7a) by the miRNA, would significantly increase their prognostic and diagnostic value.

Although data presented in previous section indicate existence of a potential association between over-expression of miR-21, decreased let-7a levels and over-expression and constitutive activation of STAT3 in HPV16-induced cervical cancer, it does not demonstrate how aberrant STAT3 signaling is involved in HPV16-mediated cervical carcinogenesis. It is also pertinent to record that the correlation study in first part of our study analyzed only the cross sectional data emerging out of cervical lesional tissues. To test clinical correlations further studies were also performed to test and support these findings with experimental data using HPV positive SiHa cervical cancer cells which show representative levels of STAT3 and miR levels to validate the observed correlation in clinical specimens. So in the next part of our study, we
modulated STAT3, miR-21, let-7a and HPV16 E6 oncoprotein expression and tested the consequences on each other.

STAT3 targeting either by commercially available siRNA or pharmacological agents as curcumin and static results in decreased STAT3 which promotes reduction of miR-21 expression in SiHa cells. miR-21 targeting favors gain of PTEN expression whereas Let-7a over-expression suppressed STAT3 in cervical cancer cells. HPV16 E6 oncoprotein inhibition increases let-7a expression with a reduction in miR-21 levels. Aberrant STAT3 expression plays an important role in cervical carcinogenesis. Our current understanding of the role of STAT3 focuses on its ability to deregulate cellular gene expression at the transcription level through its interaction with many cellular factors.

**STAT3 siRNA has blocked STAT3 at transcript level and abrogated miR-21 expression**

Transient transfection of HPV16 positive SiHa cells with STAT3 siRNA induced dose-dependent decline in expression of STAT3 with a concomitant loss of miR-21 expression. Specific targeting of STAT3 expression in cervical cancer cell lines have been performed earlier using recombinant adenoviral dominant negative STAT3 (Chen et al. 2007) or STAT3 specific siRNA (Takemoto et al. 2009) which invariably demonstrated similar decrease in cell numbers and affected the viability of cervical cancer cells. This observation prompted us to speculate that miR-21 is positively regulated by STAT3.

**Targeting of STAT3 by curcumin or static molecule results in decreased STAT3 activation and miR-21 expression**

Curcumin is derived from turmeric (Curcuma longa) and is a natural polyphenol. Curcumin has long been used as a food, coloring agent, and traditional medicine. It is safe and nontoxic and has demonstrable antitumor, anti-inflammatory, apoptotic, and antioxidant properties (Manju and Nalini 2005). Many recent evidences indicated that curcumin has anticancer effects against different types of human tumor cells, including of ovarian cancer cells, colon cancer cells and astroglioma cells (Sinha et al. 2003). Curcumin is known to inhibit STAT3 phosphorylation by EGFR, Src, and Jak2, the upstream kinases responsible for
activation of STAT3 (Bharti et al. 2003). In present study we observed that curcumin treatment results in decreased STAT3 activation at 50μM concentration and miR-21 expression was remarkably suppressed at same dose in SiHa cells. Consistent to our study, curcumin and its synthetic analog, diflourinated curcumin (CDF), either alone or in combination, down-regulated miR-200 and miR-21 expression in pancreatic tumor cells (Bao et al. 2011). Anti-cancer effect of curcumin has been demonstrated on mechanistic role of JAK/STAT3 signaling in small cell lung cancer tumorigenesis and progression (Yang et al. 2012). Stattic is a nonpeptidic small molecule shown to selectively inhibit the function of the STAT3 SH2 domain regardless of the STAT3 activation state in vitro. Stattic selectively inhibits activation, dimerization, and nuclear translocation of STAT3 and increases the apoptotic rate of STAT3-dependent cancer cells (Schust et al. 2006). We found that targeting STAT3 by stattic results in decreased activity of STAT3 in a dose dependent manner. Consistent observations were found by Pan et al. The authors showed that STAT3 was activated and they proposed that STAT3 could be blocked by Statticin nasopharyngeal carcinoma cells. The inhibition of STAT3 by Stattic decreased the expression of cyclin D1 in a dose- and time-dependent manner (Pan et al. 2013). Till now there is no report which has shown effect of targeting of STAT3 by stattic on miR-21.

**miR-21 inhibition has considerable effect on its target gene PTEN**

In the present study, transfection of miR-21 inhibitor inhibited the cell growth of SiHa cells. miR-21 inhibition by miR-21 inhibitor resulted in gain of PTEN protein levels. Our results demonstrating the negative correlation between miR-21 and PTEN levels suggests a regulatory role for miR-21 in PTEN expression. This is consistent with the findings shown in endometrial cancer by Qin et al (Qin et al. 2012). In agreement to our results, Zaman et al have shown that the miR-21 level was reduced by more than 99%, as compared to the negative control in A-498 renal cancer cells and Caki-2 cells when the expression of miR-21 was inhibited using a commercially available miR-21 inhibitor (Zaman et al. 2012). Decreased cell numbers were observed in cultures treated with anti-miR-21, compared with cultures that were incubated in regular media or transfected with a non-specific negative toxicity controlling glioblastoma-derived cell lines (Gaur et al. 2011). In another study, it was observed that anti-miR-21 decreased 70% of endogenous miR-21 expression inhuman
prostate cancer DU145 cells (Liu et al. 2011). miR-21 inhibition has been shown to inhibit the cell growth and promote apoptosis in primary tongue carcinoma and primary esophageal squamous cell carcinomas; human tongue cancer cell lines and esophageal squamous cell carcinoma cell lines (Chang, J. T. et al. 2010).

Let-7a acts as negative regulator of STAT3

STAT3 has been shown to direct target of Let-7a and new advances in miRNA research provide hope for selection of miRNAs as potent therapeutic target in cancer. To test this proposition, we over-expressed a small RNA mimic for Let-7a in SiHa cells. We found that restoration of Let-7a expression inhibited cell growth as compared to control untreated cells. In consistent with our observations, restoration of Let-7 expression has also been shown to reduce tumor growth in other cancers, such as ovarian cancer (Wang et al. 2012) and lung cancer (Esquela-Kerscher et al. 2008; Kumar et al. 2008; Trang et al. 2010; Trang et al. 2011), in which the Let-7 family is globally decreased (Takamizawa et al. 2004; Johnson et al. 2005). STAT3 expression and specifically its activation were decreased in response to Let-7a restoration in cervical cancer. This supports the negative regulation of STAT3 by Let-7a.

HPV16 oncoprotein inhibition showed increase in let-7a levels and decrease in miR-21 expression

Our study provided the finding that inhibition of HPV16 E6 oncoprotein resulted in gain of let-7a expression levels whereas E6 inhibition suppressed miR-21 at 80nM dose. This suggests that E6 might be negatively regulating let-7a but on the other hand, miR-21 might be positively regulated by HPV E6. Interestingly, specific silencing of E6/E7 using specific siRNA also results in similar growth inhibition of cervical cancer cells, loss of transformed phenotype, induce apoptosis and replicative senescence and inhibited tumor formation in animal models (Jiang and Milner 2002; Butz et al. 2003; Hall and Alexander 2003; Yoshinouchi et al. 2003; Gu et al. 2006; Sima et al. 2007; Yamato et al. 2008). Recently studies on miRs and viruses association have indicated that certain viruses could regulate miRs. Recently HIV-1 was reported to globally suppress host miRNA expression (Triboulet et al. 2007). The Epstein-Barr virus latent membrane protein 1 was reported to activate miRNA-155 transcription (Gatto et al. 2008). Wang et al have shown that hepatitis B viral
protein, HBx, also plays a role in deregulating cellular miRNAs in hepatocellular carcinoma (Wang et al. 2010). In another study HPV proteins have been shown to downregulate the levels of miR-203 expression upon differentiation (Melar-New and Laimins 2010).

In summary, we observed that specific inhibition of STAT3 levels to its protein expression along with decreased miR-21 expression levels in cervical cancer cells. In addition, targeting STAT3 by curcumin and static molecule induced decreased STAT3 expression and activation which resulted in reduced miR-21 expression levels. Inhibition of miR-21 promoted gain in PTEN expression. On the other hand, Let-7a over-expression using mimic showed suppressed STAT3 expression. Targeting HPV16 E6 oncoprotein using specific siRNA showed increase in let-7a levels whereas miR-21 expression levels were decreased. Targeting STAT3 by siRNA, curcumin and static molecule, targeting miR-21 by antisense or small-molecule compounds and may miR mimics like let-7a mimic may represent new targeted therapeutic strategies for human cancers, including cervical cancer. By regulating miR-21, STAT3 might also be involved in the regulation of other cellular events. Therefore, with respect to this aspect the significance of the present finding about the link between STAT3 and miRs is yet to be explored. Nevertheless, these studies might lead to the future development of an alternative strategy that target STAT3 and miRs in treatment of diseases, such as cancer. Thus, miR-21 and Let-7a along with STAT3 may prove useful targets for pharmacological intervention for control of cervical cancer.

Overall our study conducted on showed well association of miRs with elevated STAT3 in cervical cancer. HPV16 might be positively regulating STAT3 regulation through alteration of miR-21 and Let-7a which has been depicted in Figure DF-1. Our results, for the first time, provide an existence of a functional signaling pathway involving Let-7a, STAT3 and miR-21 which was found regulated by viral oncoprotein E6.
Figure DF1- Schematic representation showing regulation and action of miR-21 by STAT3 and HPV16-mediated effect in cervical carcinogenesis- Schematic representation showing regulation and action of microRNA-21 by STAT3 and HPV16-mediated effect in cervical carcinogenesis. The URR of HPV16 has binding sites for transcription factors including STAT3. HPV16 E6 and E7 oncogenes are situated downstream of URR. miR-21 promoter has 2 STAT3 binding sites while STAT3 3'UTR has been shown to contain Let-7a binding site. PTEN, a negative regulator of STAT3, is a direct target of miR-21. MMP-2 has been shown to contain binding site in STAT3 promoter. TIMP-3, a negative regulator of MMP-2, also has binding site for miR-21 in its promoter. When a healthy normal cell gets HPV infection, the E6 and E7 oncogenes modulate the activity of their downstream targets i.e. p53 and pRB respectively and in turn STAT3 is aberrantly regulated. STAT3 over-expression [as observed in cervical cancer, (Shukla, Bharti et al. 2009)] causes miR-21 upregulation and down-regulated of its direct target, PTEN. The resultant effect causes dysregulation of cellular mechanisms e.g., cell development, cell cycle arrest, anti apoptosis, cell invasion and metastasis which results in the production of cancer cervix cell.