1. Name of the Student: Vinayak C. Palve
2. Name of the Constituent Institution: Tata Memorial Centre, Advanced Centre for Treatment Research and Education in Cancer
3. Enrolment No.: LIFE09200704001
4. Title of the Thesis: Role of anti-apoptotic Mcl-1 gene in human oral cancers and premalignant lesions
5. Board of Studies: Life sciences
INTRODUCTION

Squamous cell carcinoma of the oral cavity (OSCC) is the most prevalent cancer in males of the Indian subcontinent, predominantly associated with the tobacco-chewing habit [1] and the absolute number of cancer deaths is projected to increase because of population growth and increasing life expectancy [2]. Despite recent advances in surgical treatment and radio/chemo therapy, the long term survival of oral cancer patients has not changed significantly. Several factors are associated with poor prognosis of OSCC. Firstly, majority of the oral cancer patients are diagnosed at an advanced clinical stage. About 40% of oral cancer patients die from uncontrolled loco-regional disease alone and 24% show metastases to distant sites [3]. Second, the development of multiple primaries has major impact on survival and outcome. Hence, it is important to elucidate the mechanisms involved in the development and progression of oral cancer. Oral carcinogenesis is a multistep process involving functional deregulation of several genes including those involved in cell proliferation and apoptosis and OSCC’s have also been repeatedly linked to apoptotic dysregulation [4]. Altered expression or mutation of genes encoding key apoptotic proteins can provide cancer cells with both intrinsic survival advantage and an inherent resistance to therapy [5].

Apoptosis or programmed cell death is controlled by a diverse range of cell signals and effected via two major pathways namely extrinsic and intrinsic. Although their differing modes of initiation are different, both pathways have common downstream events i.e. the activation of caspase cascade leading to apoptosis [6]. The extrinsic pathway is triggered by binding of extracellular ligands, oligomerization of transmembrane receptors and activation of downstream pro-caspase8 and several adapter molecules to bring above apoptosis. The intrinsic or mitochondrial pathway is triggered by various apoptotic signals like ionizing radiation, chemotherapeutic drugs disrupting the mitochondrial membrane potential with release of cytochrome-c, thereby activating with APAF-1 & pro-caspase-9 to form ‘apoptosome’ complex, which leads to disruption of cell.
The Bcl-2 family members are important mitochondrial pathway regulators and are comprised of pro- and anti-apoptotic proteins. The dynamic balance between these opposing members play a critical role in regulating cell survival [7]. Over expression of anti-apoptotic members of the Bcl-2 family like Bcl-2 & Bcl-XL, has been shown to be associated with radioresistance [8, 9]. Bcl-2 members might, therefore, function as indicators of response to radio/chemotherapy. Mcl-1 (Myeloid cell leukemia-1), is an important anti-apoptotic member of the Bcl-2 gene family, essential for development, differentiation and proliferation [10]. Elevated levels of Mcl-1 have been detected in a variety of hematopoietic, lymphoid and solid including head and neck carcinoma [11, 12]. Overexpression of Mcl-1 has been associated with poor prognosis and resistance to treatment in breast, cervical & gastric cancers [13-15].

Recent studies from our laboratory have demonstrated significant overexpression of Mcl-1 transcripts and protein in oral cancer cell lines, premalignant lesions (OSF) and tumors [16]. Further, we have also demonstrated Mcl-1 to be a prognostic factor in oral cancer patients treated with definitive radiotherapy [17]. Our earlier studies have also demonstrated a five to ten fold higher expression of anti-apoptotic Mcl-1L transcript, versus the pro-apoptotic Mcl-1S in oral tumors [16]. Mcl-1 has been shown to contribute in resistance of cancer cells to chemo/radio therapy [18, 19]. However there are no reports on role of Mcl-1splice variants in radiation induced apoptosis and radioresistance.

Therefore, in the present study we wanted to investigate the association of Mcl-1 isoforms with oral cancer prognosis and with radioresistance and or chemoresistance of oral cancer cells.
Therefore objectives of the project were-

1. To decipher mechanisms of Mcl-1 overexpression in oral cancers

2. To examine the levels of Mcl-1 isoforms in oral cell lines, premalignant lesions & paired oral tumors from different subsites and their correlation with clinico-pathological parameters.

3. To study whether Mcl-1L is a radio-resistance and/or chemo-resistance related factor in oral cancers.

MATERIALS & METHODS:

Oral tissue samples: A total of 130 patients with a diagnosis of oral cancer & 20 OSF were recruited for this study, after approval of the project by the Institutional Review Board (IRB) and with an informed consent of the individual patients. Further, 10 biopsies from patients without any clinically detected lesions undergoing minor surgical procedures like removal of third molar were collected. All the tissues were frozen immediately in liquid nitrogen and stored at -80°C until analysis.

Cell cultures: Eight oral squamous carcinoma cell lines of different origin (Tongue-AW8507, AW13516, SCC40, SCC25, SCC15, QLL1; Buccal mucosa-SCC29B; Alveolar ridge-SCC74) and one each of immortalized Fetal Buccal (FBM) & normal epidermal (HaCaT) & dysplastic oral keratinocyte (DOK) cell lines were used in study. The cells were maintained on DMEM or IMDM supplemented with 10% FBS & 1% standard antibiotic mixture in 5% CO₂ incubator at 37°C.

RNA isolation: RNA from cell pellets and tissues were extracted using TRI reagent (Sigma, USA) according to the manufacturer’s protocol. The RNA was dissolved in DEPC-treated water and contaminating DNA was removed by DNaseI treatment. RNA integrity was analyzed by electrophoresis and samples were preserved at −80°C until analysis.
RT-PCR analysis: cDNA was synthesized with 2 μg total RNA, using a First Strand cDNA synthesis kit (MBI Fermentas, Canada) according to the manufacturer’s instructions. RT-PCR was carried out using primers specific for Mcl-1 isoforms (Mcl-1L, Mcl-1S & Mcl-1ES isoforms) and β-actin.

Real time PCR: qRTPCR was performed using cDNA of cell lines and tissue samples and gene specific Taqman probes (for Mcl-1 isoforms & GAPDH) and universal master mix on ABI-7900HT Real Time PCR System (Applied Biosystems, USA). Comparative \( C_T \) method of relative quantification was used for gene expression analysis [20].

DNA isolation & PCR sequencing: The genomic DNA was extracted from cell lines and tissue samples using Blood & Tissue DNA extraction kit, as per manufacturer’s instructions (Qiagen, USA). The PCR was performed; gel products were resolved on 2% agarose gel, eluted & purified using QIAquick Gel purification Kit. The products were sequenced in automated sequencing machine and the sequences obtained were aligned with the wild type Mcl-1 gene sequences from NCBI database, using ClustalW, BioEdit, etc software’s.

Radiation Treatment: After 48 hrs of plating exponentially growing cells \((6 \times 10^3 \text{ cells})\) were treated with IR (D0 dose) using 60Co-γ radiator as described earlier [18]. Cells were incubated up to different time points, harvested and stored in -80°C until use.

Clonogenic Assay: Exponentially growing oral cells were harvested, counted and replated in duplicates. Cells were irradiated, using 60Co-γ radiator along with an untreated control. Cells were then incubated up to 14 days to form colonies which were fixed, stained and counted. The percent plating efficiency and fraction surviving a given radiation dose were calculated based on the survival of non-irradiated cells as described earlier [21].

Western blotting: Cell & tissue lysates were resolved on 12% SDS-PAGE gels and transferred onto PVDF membranes. Membranes were blocked and probed with primary antibodies: Bax, Mcl-1, Bcl-xl and β-actin. Secondary antibodies used were Horseradish peroxidase conjugated IgG. Proteins were visualized with enhanced chemiluminescence kit (GE Healthcare, US).
Densitometry analysis of developed X-ray film was performed using ImageJ software (NIH, Bethesda, MD). β-actin was used as loading control.

**Immunofluorescence staining:** Cells were grown on glass cover slips and Mcl-1 staining was performed post-IR using an Alexa fluor-488 labeled secondary antibody, as described earlier [16]. The nuclear condensation and apoptosis was analyzed by DAPI staining, cell counting and imaging was done by confocal microscope with LSM Image Browser 4.2 software (Carl Zeiss).

**Mcl-1 Knockdown:** Transient and regulated knockdown of Mcl-1 was achieved by transfection of Mcl-1L specific siRNA (sc-37007) along with a control siRNA & pTRIPZ mediated lentiviral transduction in oral cell lines. The Mcl-1 knockdown was assessed by western blotting.

**Acquired radioresistant sublines:** Radioresistant sublines were generated by irradiating AW8507 & AW13516 cells with a fractionated Ionizing radiation strategy as described [22].

**MTT assay for cell proliferation:** Cells were seeded, in triplicate in a 96-well micro titer plate in 100µl complete medium. Proliferation was studied every 24 hours up to a period of 4 days using MTT assay as described previously [23]. The growth curve was prepared from three independent experiments by plotting O.D. at 540 nm against time.

**Drug & inhibitor treatment:** In order to determine role of Mcl-1 in chemo-resistance if any, a commonly used drug, Cisplatin was used alone or in combination with Obatoclax (BH3 mimetic small molecule inhibitor) in oral cancer cells. The IC50 doses of both Cisplatin & Obatoclax were calculated by MTT assay as described above.

**Statistical analysis:** Statistical analysis was performed by using SPSS 16 software. The difference between means was considered statistically significant when P<0.05. The data is illustrated as mean ± standard deviation of three independent experiments.
RESULTS:

I. To decipher mechanisms of Mcl-1 overexpression in oral cancers-

1. Genomic alterations:

Controversial reports are available in literature suggesting a role of genomic alterations (6 & 18 bp polymorphic insertions) on Mcl-1 overexpression and disease state. In order to rule out this possibility, all three exons, two introns and promoter of Mcl-1 gene were sequenced in cell lines, healthy volunteers & paired tumor samples.

In cell lines: The sequencing analysis revealed no alterations in exons / introns of Mcl-1 gene amplified from human oral cancer cell lines. Also, sequencing of Mcl-1 isoforms (Mcl-1L & Mcl-1ES) including recently discovered Mcl-1ES, didn’t reveal alterations. Further, the promoter region of Mcl-1 was predicted using CHSL (Cold Spring Harbour Laboratory) promoter database and the entire 1.5 kb promoter region was amplified using different pairs of primers. No genomic alterations were observed in promoter region of Mcl-1 in 9 oral cancer cell lines except SCC15 & SCC40 which showed presence of additional 6 bp (GGCCC) repeat.

In healthy volunteers: The PBL’s (peripheral blood lymphocytes) were isolated from blood of 25 healthy volunteers, genomic DNA was extracted and PCR sequencing of Mcl-1 gene was carried out. Sequencing and alignment analysis revealed that 8 out of 25 volunteers (32%) showed presence of 18 bp promoter polymorphisms.

In tissue samples: Genomic DNA was extracted from 40 paired tumor tissues and sequencing was carried out. The sequencing analysis revealed no differences in presence or absence of polymorphism between adjacent normal vs. tumor tissues. Further, 9 out of 40 tumors (22%) showed presence of 18 bp Mcl-1 promoter polymorphisms. Also, two reported SNP’s were observed in oral cancer patient namely C<A-324 & G<C-386.
Effect of promoter polymorphism on Mcl-1 expression:

Real time PCR and western blot analysis of 40 normal & tumors revealed, no significant difference at both mRNA and protein level between patients with or without 18bp Mcl-1 promoter polymorphisms. Also presence or absence polymorphism does not correlate with Mcl-1L/ Mcl-1S isoform levels.

Correlation of Mcl-1 promoter polymorphisms with clinic-pathological parameters:

The $\chi^2$ square test revealed no significant correlation between Mcl-1 promoter polymorphism (18bp) and clinico-pathological parameters (Gender, Age, Habit, Site of tumor, size, Nodal status, Differentiation etc.) of oral cancer patients. Further, Kaplan Meier survival analysis also showed no significant correlation between presence or absence of 18 bp polymorphisms and overall survival of oral cancer patients.

2. Phosphorylation status of Mcl-1:

Western blot and proteosomal inhibitor treatment, revealed phosphorylation of Mcl-1 protein at Ser-159 & Thr-163 residues in AW8507 oral cancer cell line, which is essential for its proper detection by E3 ligase and further proteosomal degradation.

II. Expression of Mcl-1 isoforms and correlation with clinico-pathological parameters -

Expression of Mcl-1 isoform in oral cell lines: qRT-PCR analysis revealed, high expression of anti-apoptotic Mcl-1L isoform over pro-apoptotic Mcl-1S & Mcl-1ES in all the oral cell lines. Elevated expression of Mcl-1L was observed at both mRNA & protein level in majority of oral cancer cell lines (SCC25, SCC29B, SCC40, SCC74, QLL1, AW8507, AW13516) as compared to immortalized normal FBM, HaCaT & DOK cell lines.

Expression of Mcl-1 isoform in oral tissues:

The Immunohistochemistry analysis revealed higher expression of Mcl-1 in OSF tissues versus normal mucosa. Similarly, the qRT-PCR analysis showed an elevated expression of Mcl-1L
mRNA in 64% of oral tumors, in 130 paired samples. However, the relative expression of Mcl-1L isoform was 5 fold higher than Mcl-1S and 10 fold higher to Mcl-1ES isoform in oral tumors. Similarly, western blot analysis has revealed, five to ten fold higher expression of Mcl-1 protein in oral tumors vs. adjacent normal's.

Correlation of Mcl-1 expression with clinico-pathological parameters:

For statistically correlating the expression of Mcl-1 isoforms with clinic-pathological parameters, the data was dichotomized into two groups namely: the Mcl-1 high expressers and low expressers. For comparison the mean expression of Mcl-1 isoforms in healthy normals was used. **Univariate analysis**- revealed significant correlation of high Mcl-1L expression with node positivity (p = 0.002) and advanced tumors (p = 0.001). However, no significant correlation was observed between expression of Mcl-1 isoforms and gender, age, tobacco/alcohol habits, primary site & differentiation of s in oral cancer patients.

**Multivariate analysis**- The multivariate analysis revealed that the patients exhibiting a high Mcl-1L exhibited shorter survival as compared to those expressing low Mcl-1L. Our studies indicate that Mcl-1L expression as an independent prognostic factor for oral cancer.

Correlation of Mcl-1 expression with overall survival

The Kaplan–Meier survival curves of low and high expressers of Mcl-1L showed a statistically significant difference (p = 0.002). Moreover, patients having high Mcl-1L exhibited poor overall survival. Inversely, patients showing high Mcl-1S significantly better survival (p = 0.051) as compared to those having low Mcl-1S. The ratio of Mcl-1L/Mcl-1S, showed a positive correlation with the poor overall survival of oral cancer patients (p = 0.006). Univariate analysis also revealed poor overall survival of node positive oral cancer patients (p = 0.003). However, the other parameters like age, tobacco/alcohol habits and differentiation did not significantly influence overall survival of patients.
III. **Role of Mcl-1L as Radio / Chemo resistance related factor in oral cancers**

*In radio-resistance:*

To study the role of Mcl-1 isoforms in radiation response of oral squamous carcinoma cells (OSCC), we investigated in the present study, the association of Mcl-1 isoform expression with radiosensitivity of OSCC, using siRNA strategy.

Three to six fold higher expression of anti-apoptotic Mcl-1L versus pro-apoptotic Mcl-1S was observed at mRNA & protein levels in all cell lines, post-irradiation. Sustained high levels of Mcl-1L, downregulation of pro-apoptotic Bax & Bak and a significant (P < 0.05) reduction in apoptosis was observed in the more radioresistant AW8507, AW13516 versus FBM cells, post-IR. The ratios of anti to pro-apoptotic proteins were high in AW8507 as compared to FBM. Treatment with Mcl-1L siRNA alone or in combination with IR significantly (P < 0.01) increased apoptosis viz. 17.3% (IR), 25.3% (siRNA) and 46.3% (IR plus siRNA) and up regulated pro-apoptotic Bax levels in AW8507 cells. Combination of siRNA & IR treatment significantly (P < 0.05) reduced cell proliferation and clonogenic survival of radioresistant AW8507 & AW13516 cells, suggesting a synergistic effect of the Mcl-1L siRNA with IR on radiosensitivity. Interestingly, during the development of radioresistant sublines using FIR, high expression of Mcl-1L was observed.

*In chemo-resistance:*

To determine role of Mcl-1 as chemo resistance related factor, downregulation of Mcl-1 was achieved via cloning shRNA sequence in pTRIPZ inducible system, transduced in AW8507 cells. Another important member of anti-apoptotic Bcl-2 family, Bcl-xl was also transiently knocked down using siRNA. Western blot analysis confirmed the downregulation of both Mcl-1 and Bcl-xl proteins. Cisplatin, a common drug in treatment of oral cancer was used in the study.
The IC50 values of Cisplatin for oral cell lines by MTT assay showed that majority of oral cell lines were sensitive to Cisplatin treatment. Double knockdown of Mcl-1 & Bcl-xl in combination with Cisplatin significantly reduced cell viability & proliferation as compared to any treatment alone. Interestingly, similar results were obtained using a small molecule anti-Mcl-1 drug, Obatoclax in combination with Cisplatin exhibiting higher induction of cell death and reduction cell proliferation as compared to any treatment alone.

**DISCUSSION:**

Dysregulation of apoptosis regulating genes may play a key role in the development and progression of many human malignancies. Oral squamous cell carcinomas (OSCCs) have repeatedly been linked to apoptotic dysregulation [16]. Bcl-2 and related pro- and anti-apoptotic proteins are important mitochondrial apoptosis pathway regulators and play a critical role in regulating cell survival [24]. Earlier studies from our lab have shown Mcl-1, an anti-apoptotic member of the Bcl-2 gene family to be overexpressed in oral s [16]. Also, our studies showed together Mcl-1 with PCNA may have potential prognostic value to differentiate patients with poor DFS [17]. We have also for the first time showed the relative levels of Mcl-1 in oral s vs. adjacent normals. Overexpression of Mcl-1 in oral cancer is consistent with an up-regulation of Mcl-1 in hepatocellular carcinomas, cervix cancer, pancreatic cancer, non-small cell lung cancer, and melanomas [11]. Thus, overexpression of Mcl-1 may represent an important mechanism in the development of oral cancer.

In order to elucidate mechanisms of high Mcl-1 expression in oral tumors, we performed the sequencing of Mcl-1 gene which revealed presence of 18bp repeats in 22% of oral s. Interestingly, studies by Moshyńska et al, had shown that the presence of 6 and 18 nucleotide insertion in Mcl-1 promoter correlated with increased RNA and protein levels & clinical outcome in CLL patients [25]. The presence of insertions was shown to be associated
with poor overall survival and disease-specific survival, suggesting the potential use of the Mcl-1 promoter insertions as a prognostic factor.

To investigate the clinical significance of the Mcl-1 promoter insertions oral cancer, we analyzed 40 oral cancer patients, 10 oral cell lines and 25 healthy volunteers, utilizing PCR sequencing of Mcl-1 promoter for the presence of these two insertions. We also correlated the sequencing data with clinico-pathological parameters, overall survival & prognosis of OSCC patients. However, our data does not support the earlier findings by Moshyanska et al of positive correlation of these insertions Mcl-1 gene expression and outcome. Firstly, we have observed presence of 6/18 bp insertions in both healthy volunteers, as well as in oral cancer patients. Neither expression of Mcl-1 splice variants nor Mcl-1 protein showed a significant correlation between presence of 6/18 bp insertions in Mcl-1 promoter. The overall survival of patients was also independent of presence or absence of Mcl-1 promoter polymorphisms, indicating that Mcl-1 promoter polymorphism may not be useful to predict outcome/prognosis of OSCC patients. In support to our findings, several correspondence reports are available [26-29], indicating that the MCL-1 insertions represent hereditary polymorphisms rather than somatic mutations that likely do not predispose to chronic lymphocytic leukemia and were not associated with prognostic markers.

Studies by Reed et al, demonstrated association of between promoter insertion with high MCL-1 mRNA and protein levels, these insertions represent somatic alterations and not hereditary polymorphisms [30]. Thus, the presence of promoter insertions appears to be insufficient to reliably drive high levels of MCL-1 expression in CLL. Report from Saxena et al [31], showed that polymorphic insertions were associated with increased promoter activity & Sp1/Sp3 binding sites. However, evidence for the biological effect of MCL-1 promoter polymorphisms on gene expression and the significance of Mcl-1 promoter polymorphism in oral cancer needs to be analyzed in large cohort.
Our studies revealed a high expression of Mcl-1L in both OSF and oral tumors. To evaluate the proportion and contribution of three Mcl-1 isoforms to this overexpression in oral cancer, we analyzed the isoform levels by qRT-PCR in tumor vs. adjacent normal tissues. High levels of anti-apoptotic Mcl-1L isoform were observed in majority of oral cancer cell lines vs. normal immortalized cell lines. Also 64% of oral s showed significantly higher Mcl-1L levels as compared to corresponding adjacent normal tissues. To the best of our knowledge, there are no reports delineating the prognostic significance of Mcl-1 isoforms in human oral cancer. This is the first study demonstrating the correlation of high Mcl-1L levels with poor overall survival and its possible use as an independent prognostic marker for oral cancer patients. However, the only other of Mcl-1 isoforms in CCRCC (clear cell renal carcinoma) by Kempkensteffen et al [32], in contrast showed downregulation of Mcl-1L in CCRCC to be associated with aggressive phenotypes. Our studies revealed a significant correlation of high Mcl-1L expression with node positivity and advanced tumors. The ratio of Mcl-1L / Mcl-1S isoforms also, showed a positive correlation with the poor overall survival, indicating Mcl-1L expression as an independent prognostic factor for oral cancer patients. Our studies are consistent with the previous reports demonstrating high Mcl-1 expression to be associated with poor outcome in breast cancer, cervix cancer, gastric cancer and various hematological malignancies [13, 14, 33].

So far, limited information is available on the role of Mcl-1 in radiation response of cells. To our knowledge, this is the first study to report a time course expression of Mcl-1 isoforms post-IR and effect of Mcl-1L knockdown on radiosentitization of oral cancer cell lines using siRNA strategy. Our studies demonstrated an inverse correlation of Mcl-1 expression with cellular apoptosis and a synergistic effect of Mcl-1L knockdown along with IR on cell viability and clonogenic survival thereby enhancing the radiosensitivity of OSCC cells. Our studies revealed higher expression of Mcl-1L at both mRNA and protein level in relatively more radioresistant AW8507 & AW13516 cell lines versus FBM, indicating a possible
association of anti-apoptotic Mcl-1L splice variant with radioresistance. Moreover, it is known that the short isoform Mcl-1S only binds to Mcl-1L possibly neutralizing its anti-apoptotic function [34]. No significant alterations in levels of Mcl-1S & Mcl-1ES were observed post IR, indicating that the predominantly overexpressed Mcl-1L isoform alone may contribute in generation of radioresistance. We assessed the ratios of Mcl-1L/Mcl-1S, Mcl-1L/Bax, Bcl-xl/Bax, wherein radioresistant AW8507 & AW13516 showed high ratios as compared to that in FBM indicating predominance of anti-apoptosis which may contribute to radioresistance.

Interestingly, the combination of Mcl-1L siRNA plus IR induced significantly higher apoptosis as compared to siRNA or IR-alone in both oral cell lines. However, the expression of pro-apoptotic Bax protein correlated with the increased apoptosis on Mcl-1L knockdown. To address the fact that the induction of apoptosis may not necessarily lead to long-term response to radiotherapy, we performed the clonogenic assay which demonstrated that combination of IR and Mcl-1L downregulation synergistically reduced clonogenic survival as compared to each treatment alone. The high expression of Mcl-1L, in radioresistant sublines developed by fractionated ionizing radiation provides a direct evidence for the role of Mcl-1L in radioresistance of OSCC cells. Therefore, the combination of radiotherapy and Mcl-1L downregulation has the potential to improve the response rate of treatment resistant oral cancer cells. Selective inhibitors like Obatoclax, which specifically overcome Mcl-1 mediated resistance, is already in phase 2 clinical trials [35] and may have important therapeutic implications, when used in combination with radiotherapy in treatment of oral cancer patients.
SUMMARY & CONCLUSION:

Our studies for the first time demonstrate, overexpression of Mcl-1L isoform in oral tumors & its positive correlation with node positivity & advanced tumors. High Mcl-1L significantly correlated with poor overall survival, indicating Mcl-1L to be an independent prognostic factor for oral cancer. Our studies also indicate the association of Mcl-1L isoform with radioresistance/chemo resistance by influencing apoptosis, proliferation and clonogenic survival of oral cancer cells. Thus, Mcl-1L appears to be an important pro-survival and radio/chemo resistance factor, influencing outcome of oral cancer patients. It therefore may be a potential therapeutic target in oral cancers.

REFERENCES:


**PUBLICATIONS:**

Publications from thesis:


Signature of Student:

Date: 25/01/2013
## LIST OF ABBREVIATIONS:

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<tr>
<td>APS</td>
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