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EXPLORING DRUG-RESISTANT LONG NON-CODING RNA BIOMARKERS AND THEIR CLINICAL RELEVANCE IN HEAD AND NECK CANCER

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ABSTRACT

Introduction: Head and Neck Cancers comprise the sixth of the ten most frequent causes of deaths, and among the worst prognoses across the globe. Radiotherapy and Chemotherapy practices are considered efficient therapeutic regimes for the treatment of head and neck cancers. **Objective:** This research has also assessed the relative level of biomarkers (MALAT1, MDR1 and Caspase 8), and also forecasted their role as either drug resistance factors or indicators of cancer prognosis. **Materials and Methodology:** In this inter-collaborative study, EDTA blood of 5ml each from all the study subjects (160 patients and 40 healthy controls in addition) was taken to isolate RNA. The collected data was written down to estimate, by means of RT-qPCR, the expression level of biomarkers. **Results:** In the pathologic condition studied, there was increased expression of MDR1 in patients with Non-Hodgkin's Lymphoma but no specific change in squamous cell carcinoma patient. And this meant that the drug administered to the patients was potent. MALAT1 in down regulated with Caspase 8 and its level starts reducing in the patients who received more than 3 cycles of chemotherapy. **Conclusion:** Actually, therefore as per this study, it is assumed that if these biomarkers are investigated in relation to drug resistance, we might find a better handle on the cancer biology of H&N cancers. Therefore, leading to the development of the therapeutic approach.

Key Words: Neuro-oncology, Head and Neck Cancer, long non-coding RNA (Lnc-RNA).

INTRODUCTION

Head and neck cancer which was once regarded as the sixth most prevalent cancer in the worldwide with 350,000 fatalities per year and around 650000 are diagnosed with this particular cancer [1]. Large risk factors that are linked to various Head and Neck Cancers include Ultraviolet radiation

(particularly sunlight), Occupational carcinogens such as wood and especially the husk, asbestos and synthetic fibers, radiation whether ionizing or non-ionizing, virally transmitted diseases such as Human papillomavirus (HPV) and Epstein Barr virus [2,3]. Peripheral neuropathy, dysphagia, otalgia and some other diseases like non-healing ulcer and halitosis are among the clinical features that may be observed [4]. Head and neck targets and LncRNA biomarkers specifically against the head and neck therapeutics and prognosis were now established. Several genes use as the molecular target against head and neck cancer, which sponge with multipotent miRNAs to promote or inhibit tumor invasion, migration, and proliferation. Knowing that MALAT1 also known as NEAT2 is reported to be implicated in variety of biological processes including but not limited to gene expression, myogenesis, cell cycle regulation, protein degradation and alternative splicing [5]. Introducing, the protein MALAT1 has also been reported to be involved in metastasis and growth of one form of cancer or the other. Several research studies have shown that MALAT1 is over expressed in cancer and can therefore be used as a biomarker [6]. Recently, researchers identified that MALAT1 implicates in cell proliferation, metastasis and resistance to cell death and it also contributes to several signaling pathways. for example, MAPK; ERK, PI3K; AKT, WNT/ β catenin. MDR1 is a human gene, which codes for P-glycoprotein, a multidrug-associated protein with an intramembranous topology that exports substances out of the cell [7,8,9]. Other 4 earlier immunohistochemical and immunohistochemistry in situ analysis have also shown that MDR1 is over expressed in proximal tubules in renal cell carcinomas [10].

Other untreated malignant tumors including pancreatic tumor, colon cancer, islets tumors etc., exhibit high expression of MDR1 [11,12]. In a recent study, breast cancer cell lines were taken and Wnt / β catenin dependent luciferase assay was done, then western blotting was done to look for the expression of β catenin. From the following study it was established that Wnt / β catenin pathway has an essential role to Play in cell Cycle regulation and Cell Differentiation [13]. Aside from breast cancers, the behaviour of this pathway was found to be abnormally active in a number of other cancers and further research revealed that the Wnt / β catenin pathway activity was also shown to increase the extent of MDR1 [14]. When Caspase-8 is low or blocked significantly leads to apoptosis suppression, and the switch to the necrotic mode of death [15,16]. The latter rather promoting apoptosis and the former survival, it is known that a specific interaction with the pseudo-caspase cFLIP is responsible for the control of the activity of the protease and that the heterocomplex formed by these two partners is in charge of shifting the substrate specificity of caspase-8 in order to inactivate components of the RIP kinase pathway [17,18]. The goal of the study was to understand the meaning of the statistically significant association between the number of biomarkers that were expressed in the context with the study subjects' status and their application in the future.

MATERIALS AND METHODS

5ml EDTA blood was collected from each of 200 study subjects (160 patients, 40 Healthy Controls) at different time points of chemotherapy from Department of Oncology at Three major Hospitals in Islamabad (PIMS Hospital)and Karachi (Agha Khan Hospital, Cancer Foundation Hospital). Sample were transported to affiliated Molecular Diagnostic Labs under WHO standard STPs sample transportation protocols. **Inclusion criteria** consisted of the patients aged between 20 and 80 years, Patients diagnosed with head & neck cancer, Patient who signed the informed consent and fulfilling all clinical conditions. Patient who didn't give informed consent, Patients treated for other cancers were excluded. RNA was extracted using (Invitrogen TRIzol reagent: Catalog #15596026, USA) as per manufacturer's instructions. Quality and quantity was checked by Nanodrop 2000/2000c spectrophotometer (Thermo Scientific). Only RNAs giving 260/280 and 260/230 ratio more than 1.5 along with depiction of clear bands on agarose gel electrophoresis were processed for cDNA synthesis. Otherwise, re-precipitated or extracted again. cDNA was synthesized by using a Thermo Scientific kit (RevertAid First Strand cDNA Synthesis Kit: Catalog #K1622, USA) as per the manufacturer's protocol. RT-qPCR was performed on CFX 96 qPCR Bio-Rad, USA to measure the expression levels of mRNA of *MALAT1, MDR1* and *Caspase 8*. It was done by using (ThermoFisher Scientific SYBR Green qPCR Master Mix: Catalog# 4309155, USA) as instructed by the

manufacturer. Significant relation between two variables was assessed by unpaired t-test & for three variables one way ANNOVA with 95% confidence intervals. Results were expressed as mean ±SD). All the experiments were performed multiple times and showed consistent reproducibility. A p-value of ≤0.05 was considered as statistically significant. For statistical analysis Graph Pad Prism Software (version 9) was used and presented by bar charts.

RESULTS

Demographics

In this inter-collaborative conducted study 160 patients of head and neck cancer (HNC) were enrolled with 40 healthy controls. The age range of 10% study subjects was between 18-40 years and 90% of the patients were between 40-80 years. The ratio of female to male was 1:3 with standard deviaiton of + 8.35 and + 9.96 respectively. The division of patients on the basis of chemotherapy cycles was as 40% (cycle 1-3) and 60% (cycle 4-6). Present study divided subjects into groups for the comparative analysis between diseases patients and healthy persons **as shown in Table 1**.

Table 1: Tumor assessment of Head and Neck cancer Patients

Tumor stages	**Cases (%)**
Stage 3	45%
Stage 4	55%
Tumor subtypes (Groups)	
Squamous cell carcinoma (SSC)	72%
Hodgkin Lymphoma (HL)	7%
Non-Hodgkin Lymphoma (NHL)	7%
Pleomorphic Adenoma (PA)	7%
Ewing's Sarcoma (ES)	7%

There was stable expression of MDR1 gene in squamous cell carcinoma (SCC) and pleomorphic adenoma (PA). This means, that these cancer types are sensitive to the chemotherapy drugs which the patients were receiving namely, Cisplatin. Moreover, Non- Hodgkin's Lymphoma (NHL) and Ewing's Sarcoma (ES) showed moderately elevated expression level of MDR1 as compared to other control and types **as shown in Figure 1**. However, the levels of MDR1 in Hodgkin's Lymphoma were also seen to be non-significant. These results further explain that the chemotherapy drugs used for these cancer types were not that much effective. Hence, resistance to the drugs given can be predicted.

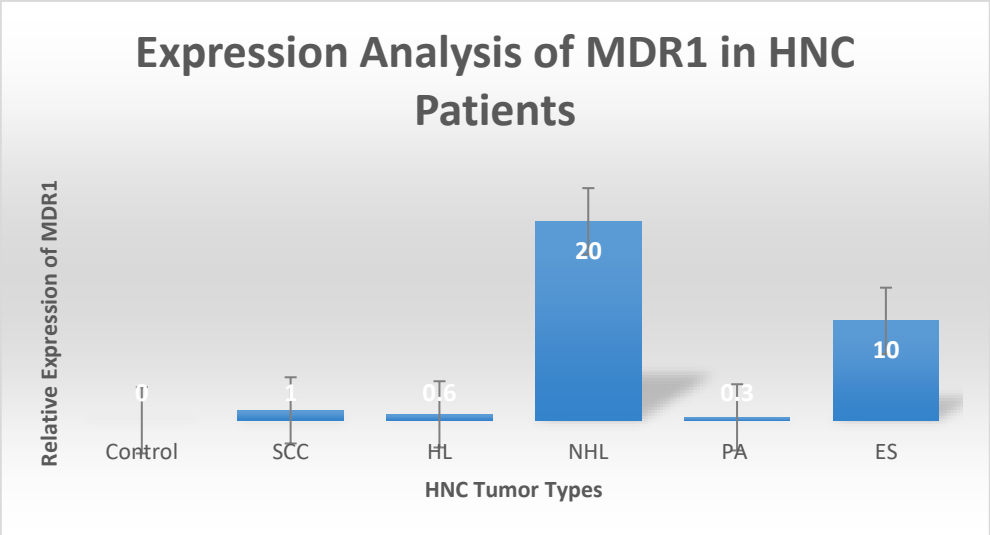

Figure 1: Relative Expression of MDR1 in Controls and Head and Neck Cancers

As the chemotherapeutic treatment advanced, the patients receiving the treatment showed less resistance to drugs. According to the cut off value which is 0.5, our results showed that the patients who received less than three cycles of chemotherapy showed elevated levels of MALAT1. Surprisingly, patients who received more than three cycles of chemotherapy showed remarkably non-significant levels of MALAT1. Therefore, our results showed that decreased levels of MALAT1 were associated with the effectiveness of the drug given along with decreased cancer cell proliferation strength **as shown in Figure 2**.

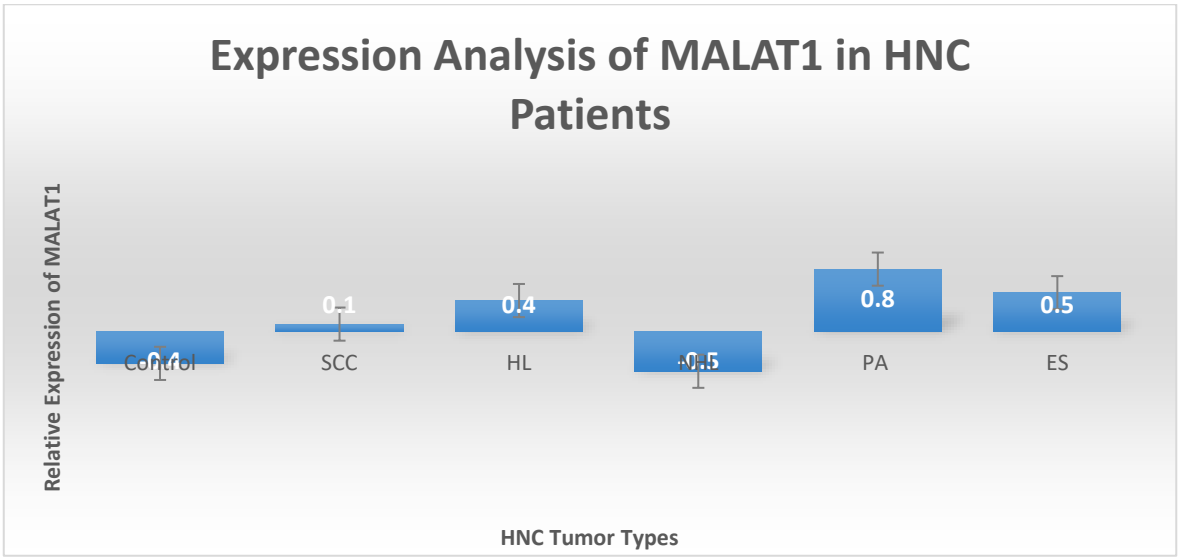

Figure 2: Relative Expression of MALAT1 in Controls and Head and Neck Cancers.

The expression of Caspase 8 in Hodgkins Lymphoma (HL) patients was seen to be significantly increased as compared to that of controls. Whereas, Caspase 8 expression remains stable in squamous cell carcinomas, Non- Hodgkins Lymphoma and Pleomorphic Adenoma, as compared to controls. Moderately increased levels of Caspase 8 were seen in patients with Ewing's Sarcoma **as shown in Figure 3**.

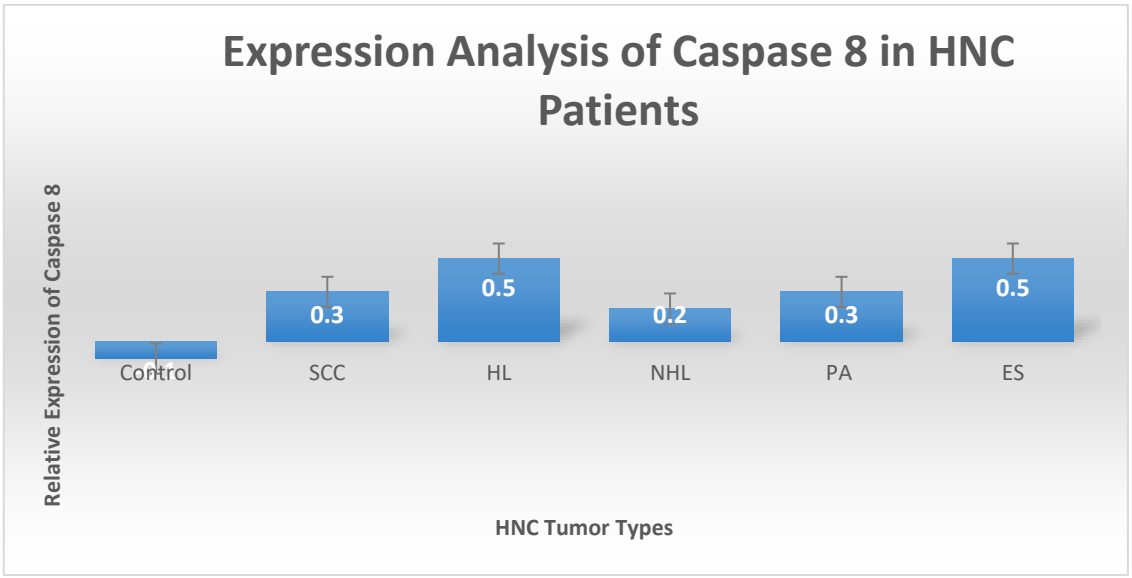

Figure 3: Relative Expression of Caspase 8 in Controls and Head and Neck Cancers

DISCUSSION

This study implies that long non-coding RNA (lncRNA) biomarkers are important in the field of drug design against cancer and sustaining their effects. However, our research shows that as a group,

MDR1 (multidrug resistance protein 1) is expressed at relatively consistent levels across several different cancers, making it a promising treatment target, but the MDR1 gene's transcription is more complicated than that of other proteins, and several transcription factors and repressors are involved in its regulation. The relationship between MDR1 expression and chemotherapeutic responses has been shown to provide valuable information in the diagnosis and prognosis of head and neck cancers (HNCs) that include squamous cell carcinoma of the head and neck (SCCHN) [19]. Several MDR1 upregulating oncogenic transcription factors have been demonstrated to function in HNC: hypoxia-inducible factor 1 alpha (HIF-1α) [20,21]. However, even to this day, no one knows (or at least indicates) exactly by what means MDR1 protein expression is controlled, and this is a crucial area for future research to resolve.

This is because given that MDR1 is a translocator for a large number of cytotoxic agents including paclitaxel, doxorubicin, and vinblastine, the intracellular concentration of these drugs decreases resulting in decreased therapeutic effectiveness [21]. This agreement with previous results in patients with Hodgkin lymphoma, where patients initially responded well to chemotherapy, but then became resistant over time because of overexpression of MDR1 [22], is further supported by these results. Like MDR 1, increased levels have been associated with resistance to platinum-based chemotherapy in other cancers [23].

Furthermore, our results suggested that besides MDR1, MALAT1 (Metastasis Associated Lung Adenocarcinoma Transcript 1) is highly expressed in several types of HNCs, mainly in the advanced disease stage [24]. The action of MALAT1 is a critical part of cancer metastasis and overall prognosis. Elevated levels of MALAT1 in patients are associated with poor survival compared to those with low levels, suggesting that MALAT1 may serve as a type of prognostic biomarker for HNC [25]. Interestingly, in our study, patients with oral squamous cell carcinoma (OSCC) in the advanced stage did not show significant changes in MALAT1 expression, which is contrary to what we expected. This suggests that MALAT1 plays a different role in different types of cancer at different stages, something that needs further targeted research.

A recent study spotting MALAT1 expression across different stages of esophageal cancer found a correlation between cancer stage and MALAT1 expression, which once again confirmed that MALAT1 could be a prognostic marker for cancer [26]. We find that MALAT1 expression is reduced as the disease advances, whereas these findings are at odds with it. The heterogeneity of cancer types and the intricate functional gene expression networks that are involved during cancer disease progression can contribute to this opposing trend.

Additionally, it has also been shown that silencing MALAT1 can make NPC cells radiotherapy responsive, both in vitro and in vivo [27]. These findings support the idea that MALAT1 is involved not only in cancer progression but also in resistance to treatment. Since MALAT1 is such an important target in HNC treatment it could improve patient outcomes for those with advanced disease with radiotherapy. Research in squamous cell carcinoma (SCC) with both antibodies and siRNA against AURKA (Aurora kinase A) has also shown that inhibition of the AURKA enhances the survival and prognosis of animal models, adding to the idea that molecular targets may be able to change the course of HNC treatment strategies [28].

Caspases play another vital role in HNC treatment, they are part of apoptosis. In contrast to conventional cancer therapy, caspase inhibition has not been widely explored due to the desire generally to induce cell death, not to block it [29].In contrast, the current literature reveals a unique feature of CASP8 where it has been discovered to mediate the increased sensitivity of HNSCCs to treatment [30]. Caspase-3 and Caspase-9 were also noted for elevation of tumor promginitive biomarkers in OSCC; positive correlation between Caspase-3 and prognosis of OS has also been reported [31]. These results imply that the proapoptotic caspases' functions in cancer biology are not as straightforward as once believed and that additional work is required to identify the potential of caspases for cancer treatment. The other finding of interest in this study is on the link between HIF-1α and cancer cell survival. The down regulation of ROS and consequent increase in cell survival observed in Hodgkin lymphoma (HL) cells with small cell size and high HIF-1α expression [32].

Therefore, HIF-1α can be considered to be a cancer specific target molecule with possibilities of using anticancer agents to modulate ROS levels for targeted cancer cell killing. Since HIF-1α also activates MDR1, therefore, targeting this pathway may help to overcome drug resistance in HNCs [33].
They further mentioned that drug resistance as well as cancer recurrence pose a major problem in the clinical treatment of HNCs. Consequently, MDR has been described in various HNC patients with disease recurrence and progression [34]. This resistance is often linked to high levels of MDR1 protein, which pumps out the chemotherapuetic drugs before they can accumulate in the tumour cell population and destroy it. MDR in HNC patients prognosticates cancer recurrence, and studies in oncology have demonstrated a favorable relation between reducing MDR1 expression and conquering drug resistance [35]. Last but not least, long non-coding RNAs (lncRNAs) have been recognized as the novel and potential marker/targets in cancer. Besides MALAT1, there are many other lncRNAs listing as novel that play important roles for cancer progression, drug resistance and treatment response [36]. For example, lncRNAs can function as molecular sponges for miRNAs sequestering them away from their target mRNAs and therefore regulate gene expression post transcriptionally [37]. Because lncRNAs have the ability to modulate gene expression, they are attractive targets for cancer therapy, especially in HNCs where therapeutic resistance and recurrence are common. Several research articles focusing on lncRNAs have proven that they do participate in the modulation of crucial signaling pathways in the field of cancer biology. For instance, lncRNAs have been shown to impact Wnt/β-catenin, MAPK, and PI3K/Akt pathways which all are important in cancer cell proliferation, migration, and survival [38]. The new frontier in the HNC treatment involves targeting these pathways through lncRNA-mediated mechanisms [39].
Other recent advances in immunotherapy also paint lncRNAs in a larger role concerning the immune response in cancer [40]; lncRNAs can affect immune cell activity in the tumor microenvironment and thereby influence tumor progression as well as immunotherapy response [41]. For HNC, this is especially relevant as immunotherapy is becoming an important part of treatment regimens in more advanced-stage patients [42]. These results suggest that increasing our understanding of the interplay between lncRNAs and the immune system may result in even more powerful combination therapies for HNC patients.
Finally, we find that MDR1 and MALAT1 have crucial roles in HNC progression and resistance to treatment. Both biomarkers play a critical role in key pathways that regulate the survival, metastasis, and therapy response of cancer cells. One way to develop more effective HNC treatments would be to target these molecules, along with other lncRNAs. Future research should determine the specific mechanisms through which these biomarkers influence cancer biology and assess the possibility of them as therapeutic targets. Furthermore, a mechanistic relationship between MDR1 expression and chemotherapeutic response is warranted, as such a relationship may form a basis for more personalized treatment strategies for HNC patients. Knowing how to reverse drug resistance and cancer recurrence will inform next-generation therapeutics to combat HNC that are targeted and effective.

LIMITATIONS OF CURRENT RESEARCH:

Despite the progress in understanding long non-coding RNAs (lncRNAs) like MDR1 and MALAT1 in head and neck cancer (HNC), significant challenges remain:

- **Heterogeneity of Cancer Types:** Tumor heterogeneity complicates the generalizability of findings, as lncRNA roles may vary across cancer stages and subtypes.
- **Lack of Mechanistic Insights:** Mechanisms regulating MDR1 and MALAT1 expression remain poorly understood, limiting targeted therapeutic strategies.
- **Clinical Translation:** Translating preclinical findings into effective clinical interventions remains a hurdle due to variability in therapeutic responses.
- **Treatment Resistance:** Cancer recurrence and resistance due to MDR1 and MALAT1 require more research to identify strategies to overcome these challenges.

- **Ethical and Safety Concerns:** Targeting lncRNAs raises concerns about off-target effects and long-term safety in cancer therapy.

CONCLUSION

MDR1 showed no significant results, which means the drugs used for treatment were effective enough. An inverse correlation between MDR1 and Caspase 8 was also observed in different Head and Neck Cancers showing that greater levels of drug resistant MDR1 gene can lead to decreased apoptotic activity. This study would provide a platform for the formulation of personalized therapeutic regimes for patients suffering from head and neck cancers. This would help develop effective ways of treatment and poor recurrence rates. This study was limited by the small sample size. In future, we hope to touch different aspects related to head and neck cancers in different time lines i.e. before, during and after chemotherapy cycles by plan to do an extensive study extending from 10 to 12 months, which would give us enough time to follow up patients.

FUTURE PERSPECTIVES:

- **Enhanced Biomarker Validation:** Conduct robust studies to validate lncRNAs like MALAT1 and MDR1 as reliable biomarkers for HNC.
- **Innovative Therapeutics:** Develop siRNA-based or CRISPR-mediated therapies targeting specific lncRNAs to combat drug resistance.
- **Personalized Medicine:** Incorporate lncRNA profiles into personalized treatment plans for improved outcomes.
- **Combination Therapies:** Explore integrating lncRNA-targeting therapies with immunotherapy or radiotherapy to enhance efficacy.
- **Ethical and Regulatory Frameworks:** Establish guidelines for safe and ethical application of novel lncRNA-based interventions.

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