Head and neck squamous cell carcinoma (HNSCC) is the sixth most common cause of cancer mortality in the world. Some progress has been made in the therapy of HNSCC, however treatment remains unsatisfactory. Recent studies have shown that different types of long non-coding RNAs (lncRNAs) are dysregulated in HNSCC and correlate with tumor progression, lymph node metastasis, clinical stage and poor prognosis. lncRNAs are a class of functional RNA molecules that can not be translated into proteins but can modulate the activity of transcription factors or regulate changes in chromatin structure. The lncRNAs might have potential of biomarker in HNSCC diagnosis, prognosis, prediction and targeted treatment. In this review we describe the potential role of lncRNAs as new biomarkers and discuss their features including source of origin, extraction methods, stability, detection methods and data normalization and potential function as biomarkers in HNSCC.

Key words: HNSCC, biomarkers, lncRNA, head and neck.

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InCpRNA in HNSCC: challenges and potential

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Head and neck cancers

Head and neck squamous cell carcinoma (HNSCC) including tumors that occur in the oral cavity is the sixth most common cancer and one of the most common cause of cancer mortality worldwide. Tobacco smoking, alcohol consumption and human papilloma virus (HPV) or Epstein-Barr virus (EBV) infections are the main causes of these malignancies. HNSCC often develops within pre-neoplastic fields of genetically altered cells. HNSCC is divided into many types according to tumor localization: tongue squamous cell carcinoma (TSCC), oral squamous cell carcinoma (OSCC), laryngeal squamous cell carcinoma (LSCC) and nasopharyngeal carcinoma (NPC). For the last decade it has been mainly treated by surgical resection, radical radio(chemo)therapy or systemic treatment alone (e.g. cetuximab, cisplatin, 5-fluorouracil or taxanes). The 5-year survival rate has persisted at approximately 43%. The most cases are not early diagnosed until cancer metastases to the regional lymph nodes of the neck what influences on the patients’ survival rate [1–4].

Recently pembrolizumab (anty-PD1) has been approved by the U.S. Food and Drug Administration in the second line treatment in patients with advanced HNSCC [5]. However, treatment results remain unsatisfactory despite these efforts. A high proportion of patients who do not respond to standard treatment could get a benefit from personalized therapy based on the molecular diagnostic or targeted therapies. Many studies have shown abnormal changes of many types of RNA (coding and non-coding) in HNSCC patients, which have pivotal role in the cancer biology. This suggests that coding and non-coding RNAs can serve as biomarkers for treatment response prediction or as diagnostic tools [1, 6–8]. However, the role of long non-coding RNA (lncRNA) are still not deeply understood.

lncRNA – biogenesis, function and role in cancer

Long non-coding RNAs are a class of functional RNA molecules that are not translated into proteins and consist of at least 200 nucleotides [9, 10]. However, this remains debatable, and some scientists postulate that lncRNAs have limited protein-coding ability – some transcripts can function in dual roles both as coding and non-coding RNA [11, 12]. It is thought that 93% of the human genome can be transcribed into RNAs [13]. Approximately 2% of these transcripts will be translated into proteins, and the remain-
ing 98% – called non-coding RNAs (ncRNAs) – will rarely be transcribed [14].

The ncRNAs are classified into two groups. The first group is called constitutive RNA and it includes transfer RNAs (tRNAs), ribosome RNAs (rRNAs), small nuclear RNAs (snRNAs) and small nucleolar RNAs (snoRNAs). The second group, called regulatory RNAs, consists of small interfering RNAs (siRNAs), piRNAs, microRNAs (miRNAs), natural anti-sense transcripts (NATS), circular RNAs (circRNAs) and long non-coding RNAs (lncRNAs) [15–17]. All ncRNAs, except for tRNAs and rRNAs, are considered “transcriptional noise” [18]. However, lncRNAs are highly transcribed and believed to play roles in more complex biological functions, i.e. regulation of gene expression at the transcriptional level in nucleus (chromatin regulation, alternative splicing of pre-mRNA, DNA demethylation and nuclear organization) or posttranscriptional level in the cytoplasm [18]. It should be noted that large proportions of lncRNAs are closely connected with genes encoded near specific mRNAs and these ‘lncRNA-mRNA pairs’ influence of each other [19, 20]. Guigo’s group reported that lncRNAs exhibit unusual exonic structure and can be alternatively spliced [21]. Another classification of ncRNAs is based on their position relative to protein coding genes: intergenic, intragenic/ intronic and anti-sense [9].

The location in the genome (overlapping with possibly important genes) and difficulties in finding an appropriate model make functional study of lncRNAs challenging. The choice of a suitable model is a problematic issue because of the lack of conservation at the nucleotide sequence level [16], tissue specific expression level [22], transcription initiation from regions rich in repeats [23] and mostly high isoform heterogeneity. The lncRNA isoforms can have different functions [24]. Moreover, recent studies have shown that lncRNAs may have cell-type specificity [25], and their function should be verified in different cell models.

Despite the fact that the vast majority of long non-coding RNAs remain functionally uncharacterized, some of them have been linked with a range biological processes including chromatin modification, regulation of transcription factors, mRNA processing and degradation as well as cell signaling [26]. They also have a vital role in cellular processing, and their deregulated expression has been associated with different types of cancers [27, 28]. Even though many cancer gene-profiling studies have revealed some cancer-associated lncRNAs, there are very few lncRNAs reported for HNSCC.

Features of lncRNA molecules and methods of analysis

Early diagnosis of cancer results in more effective therapy, and biomarkers to predict and monitor treatment response are urgently needed [29, 30]. The use of DNA or RNA as a potential biomarker is not innovative, but there are not many DNA or RNA-based markers translated to clinics. Detection of abnormal expression of lncRNAs from tissue, blood or urine samples seems to be easily performed using molecular biology methods nowadays [27, 31, 32]. An ideal biomarker should be simple obtained from the diverse of sources and require simple measurement methodology [33]. However in the case of lncRNAs some problematic questions have arisen and they need to be clarified before implementation of these molecules in the clinical diagnosis.

First of all, it is thought that good biomarker should be easily available. lncRNAs are present in tissue, peripheral blood, serum, saliva, urine or some cell-derived exosomes [31, 32, 34, 35–37], but not all lncRNAs are present in every type of biological material. For example, Tang et al. observed the presence of HOTAIR, HULC, MALAT1, MEG-3, NEAT-1 and UCA1 in malignant and adjacent nonmalignant samples from OSCC patients, but in saliva only HOTAIR and MALAT1 were detectable [36].

High quantity and quality of biomarker molecules are also important. lncRNAs are supposed to be less stable and easier to degrade than miRNAs due to their length. However, Kraus et al. showed in their studies of postmortal brain tissues that some lncRNAs are more stable than miRNAs [38, 39]. Others also indicated that most lncRNAs are stable (half-life more than 16 h) – especially intragenic and cis-antisense lncRNAs compared with those derived from introns [10, 36]. The specific lncRNA half-life depends not only on its coding place in the genome and posttranscriptional modifications but also on subcellular localization and function [10]. Moreover, the presence of some lncRNAs in body fluids such as saliva [36], and resistance of plasma lncRNAs to RNase A digestion and overnight incubation at room temperature [37] confirm high stability of these transcripts. On the other hand, analysis of both long coding and non-coding RNA transcripts obtained from archived formalin-fixed paraffin-embedded (FFPE) blocks is difficult because of their low stability [40]. However, this problem can be solved by measuring the expression level of lncRNA by real-time PCR reaction with three different pairs of non-overlapping primers [41].

The next issue refers to the standardization of material sample and the RNA isolation method. There is a lack of specific methods to sample and store material and methodological differences occur. One can compare cancer tissue with adjacent non-cancer samples from the same patient or with samples from healthy donors without a history of cancer. In our opinion, adjacent non-cancer samples are not good reference because of the risk of tumor influence or inflammation. There is also a lack of RNA isolation methods dedicated to lncRNA analysis. lncRNA from tissue and cell lines is usually extracted using standard methods for total RNA isolation based on classical TRIzol or column-based methods [42]. The isolation method seems not affect lncRNA quantification results, but there is no available data supporting this statement. However, column-based approach seems to be better than TRIzol extraction especially in the case of RNA extraction from body fluid [43]. For circulating lncRNA, the sample choice, handling and processing as well as contamination of blood cells influence the sample preparation. Due to coagulation and hemolysis, blood cells release lncRNAs into the serum affecting the results [37, 43, 44]. However, the use of special blood collection tubes can minimize the
level of background RNA and eliminate false results under quantification [45].

The detection of IncRNA, its quantification and determination of the transcriptional activity of the IncRNA gene (methylation) should be also considered. There are many methods to determine these: i) IncRNA immunoprecipitation; ii) IncRNA in situ hybridization; iii) Au-NP assay (gold nanoparticle-based); iv) IncRNA northern blot analysis; v) methylation status using HRM (High Resolution Melting); vi) microarray or RNA sequencing; vii) and the most widely used qRT-PCR or new developed ddPCR [31, 32, 46–48]. The choice of proper analysis method depends on kind of study (screening or specific detection), type of material source and costs.

The most common methods in IncRNA studies are hybridization assays especially qRT-PCR. The available qRT-PCR IncRNA platforms allows simple and quick quantification of 90 IncRNAs based on CT analysis in one run, while one commercial IncRNA microarray platform can check the expression of more than 30,000 IncRNAs without sophisticated bioinformatics methods required for NGS (Next Generation Sequencing) data extraction [49, 50]. Moreover, microarray experiments seem to be more precise because of the well-validated technology, in contrast to lab-designed qRT-PCR primers, which can differ among laboratories [51]. In addition, the presence of IncRNA isoforms and their polymorphisms influences the function of specific IncRNA [52–54], but there is a lack of information about specific studies. This can make it difficult to compare results.

Microarray or NGS methods are expensive and data analyzing is demanding and probably they will be used only in biomarker research area. The simplicity of performing and low cost as well as availability of IncRNA quantification kits with well-defined workflow seem to make qRT-PCR as a gold-standard of IncRNA quantification.

The most popular qRT-PCR method used in IncRNA research is based on SYBR-Green dye and TaqMan probes [42]. qRT-PCR assay requires the right choice of cDNA synthesis method and the proper reference genes. There are no standardized methods for reverse transcription of IncRNA. Some IncRNAs have endogenous polyA tails but others do not possess these elements. Moreover, most IncRNA is present in low copy numbers, and this makes it difficult to quantify with conventional methods. These IncRNA require the addition of polyA tails and annealing anchor dT adapters before cDNA synthesis. This approach allows enhancement specificity and sensitivity of IncRNA quantification [55]. However, in most studies, the cDNA is prepared using kits containing mixtures of oligo(dT) and random hexamer primers.

Another very important issue is the preparation of RNA to cDNA synthesis, particularly circulating RNA. Qi et al. noted that quantification of circulating RNAs via the NanoDrop spectrophotometer is problematic. They recommended using the same volume of input rather than the same amounts of RNA. However, they showed no evidence supporting this statement [43].

The use of proper reference gene is still problematic in IncRNA expression measurement using qRT-PCR. There is a lack of standardized references, and most IncRNA studies are based on GAPDH, U6 (RNU6B) or ACTB [42]. The mismatches reference influences the results and makes it difficult to compare various studies. The problems with the normalization were observed in the case of miRNA expression studies. The snoRNAs used as normalization for miRNAs are not stably expressed and actually could serve as prognostic factors [51]. This situation suggests that proper normalization is an important step in data presentation and comparison. It should be verified if different types of tissue need specific normalization genes for examination of IncRNA. For example, different IncRNA references are suitable only for brain tissue studies. Some can be applied as universal references in profiling various gliomas and normal tissues [38, 39]. Thus, it should be verified if different cancers need specific IncRNA-related normalizing genes [43]. Fang et al. checked 16 different reference genes regarding cancer, normal and metastasis tissues (such as ACTB, TUBA3, KALPHA1, GAPDH or B2M); for example ACTB was selected as the best normalizer for MALAT1 [55]. One of the open questions is normalization of circulating IncRNA data. Dong et al. verified the utility of ACTB, GAPDH, HPRT, 18S RNA, CYC, and GUSB as the reference genes in the serum of healthy and cancer patients, and ACTB was selected as the best normalizing gene. Moreover, ACTB is stable after temperature changes in serum samples [56].

Despite the numerous problems, IncRNA under proper laboratory conditions seems to be a good candidate as biomarker and this statement is proven by many diagnostic studies [43]. The features of IncRNA molecules as biomarker were summarized in Fig. 1. However, standardization of procedures and definition of specific expression profiles bearing specific clinical information before using IncRNA as biomarkers are urgently needed and are the challenge for the future studies.

**IncRNA biomarkers in HNSCC**

Many studies have shown that deregulated expression of IncRNAs can be associated with diabetes [57], leukemia [58], solid cancers [59] or other diseases such as endometriosis [60]. The potential role of IncRNA as biomarkers in cancer such as gastric, colorectal, prostate, lung cancer or HNSCC has already been described [27, 42]. While HOTAIR is deregulated in many cancer types, a few IncRNAs are deregulated in a particular cancer type, i.e. prostate cancer antigen 3 (PCA3) is found only in prostate tissue [61]. The results are promising because PCA3 may be potentially used as a cancer-type specific biomarker.

To date, only nine independent studies show global analysis of IncRNA expression profile in HNSCC: three bioinformatic analysis of available data, four experimental microarray studies and two experimental next generation sequencing studies (Table 1).

Profiling studies revealed that specific IncRNAs expression in cancer tissue is associated with HPV status, known mutations, cancer-related pathways and gene copy number changes [62–66]. We need to remember, that specific global expression analysis is based on the use of some bioinformatics tools [49, 50] and the results should be ver-
alleles by different methodologies. Thus, most of these studies are not validated using different types of samples or in vitro models. Moreover, the inaccuracies of the results may be caused by differences in examined groups or samples (such as anatomical sites) reflecting genetic diversity. Biological role of only a few lncRNAs dysregulated in HNSCC is well known. The most studied lncRNAs both in vivo and in vitro in HNSCC are HOXA13, HOXDL1, UCA1, LET, MEG3, MALAT1, H19 and NAG7. They are involved in many important cellular processes such as proliferation, migration and invasion, apoptosis or phenotype regulation. Their exact function in biology of HNSCC has been carefully described by us elsewhere [67].

However, some lncRNAs have a strong prognostic ability for overall survival, disease-free survival, or recurrence-free survival in HNSCC. These lncRNAs described as potential biomarkers are presented in Table 2. Some of lncRNAs are proposed to be independent of gender, organ site, tumor stage or TP53 status [64, 66, 68]. lncRNAs can also be used as virus infection indicators [63], while others may serve as metastasis or disease progression markers [69].

Unfortunately, there is only one study indicating lncRNA as biomarkers related to response to chemoradiotherapy. Fayda et al. showed that only plasma circulating GASS might be a useful predictive biomarker [35]. However, this study is based only on a small group of patients, and these results should be verified in a randomized trial before clinical use [43]. However, expression of lncRNA changes after exposure to chemotherapeutic drugs, and this could maintain drug resistance or sensitivity in cancer cell lines [70–72].

Only one study has indicated a role of lncRNA in radioresistant NPC cell lines. Li et al. used NGS technology and showed some previously known and some novel lncRNAs are dysregulated in radioresistant cell (Rs) line compared to the parental line. Three pairs of lncRNA-mRNA in CNE-2-Rs and 6-10B-Rs cell lines have been described. The discovered lncRNAs: n373932, n409627 and n386034, regulate SLITRK5, PRSS12 and RIMKLB mRNAs, respectively. Only CNE-2-Rs cell lines show a slight change in lncRNAs-mRNAs. In the case of 6-10B-Rs, there is strong down-regulation of n373932 lncRNA and up-regulation of SLITRK5 mRNA. Moreover, the expression of n373932 and SLITRK5 is negatively correlated in NPC patients [73]. More in vivo and in vitro studies are needed to define exact role of specific lncRNA in chemo- and radioresponse and next validation of predictive lncRNA panel in clinical practice.

Conclusions and future perspectives

lncRNAs, including the ones described here, are aberrantly expressed in a number of different cancers and were characterized as molecules with a great impact. These recently discovered RNA molecules affect the hallmarks of carcinogenesis including proliferation, metastasis and apoptosis. Moreover, existing reports indicate the potential role of lncRNA as a new class of biomarkers. However, there are some challenges and problems to take and solve before use. First of all, there is only a few data regarding specific lncRNA in HNSCC. Despite the fact that several studies have shown global changes in lncRNA expression profile in HNSCC, the validated panel was not proposed. Moreover, most studies are based on a small study group, and it is difficult to compare them to TCGA-based analyzes. Some of lncRNAs described in this review play pivotal roles in HNSCC and might be biomarkers of treatment response. However, only a few studies have focused on lncRNA after irradiation or chemoexposure. The exact role of specific lncRNAs in regulation and response to radiation and chemotheraphy used in HNSCC is unknown and requires future studies. The next issue is lack of well-validated methodology to use lncRNA in diagnostics such as different detection methods as well as use of non-specific or unsuitable normalization genes, which influence on the results.
We supposed, that future cancer diagnostic panels will likely consist of a wide variety of genes—both protein coding and non-coding. Such a platform will offer a detailed description of the nature of the tumor, and this will allow personalized treatment of HNSCC.

The examples presented here are just the tip of the iceberg. Knowledge about lncRNA is evolving. A lot of work was done to explain the role of these molecules in the cancer biology, but much more should be done to use this knowledge in clinical practice.
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### Table 2. lncRNAs described as potential biomarkers in HNSCC

| lncRNA     | Description                                                                                                                                                                                                 | Ref. |
|------------|-------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|------|
| NEAT-1     | – Significant up-regulated only in metastatic OSCC samples; not found in patients’ saliva<br>– Over-expressed in LSCC                                                                                                      | [62, 36, 75, 76] |
| HOTAIR     | – Over-expressed in LSCC samples; correlated with poor differentiation cancers, lymph node metastasis, resistance to apoptosis and more advanced clinical stage<br>– Over-expressed in OSCC of metastatic and non-metastatic tumors; correlated with lymph node metastasis, tumor size, clinical stage and histological differentiation; associated with poor OS and DFS<br>– Increased expression of exosomal HOTAIR and miR-21 in LSCC patients' blood, correlated with clinical stages, T classification and lymph node metastasis; miR-21 and HOTAIR can discriminate the patients who are at the risk of developing LSCC<br>– Independent prognostic marker for patients’ progression and survival in the NPC patients | [36, 77–81] |
| HOTTP      | – Over-expressed in TSCC samples; associated with clinical stage, tumor size, distant metastasis and patients’ OS; an independent poor prognostic factor                                                                 | [32] |
| UCA1       | – Over-expressed in TSCC samples; correlated with lymph node metastasis – potentially prognostic indicator of lymph node metastasis<br>– In OSCC samples lack of differences in expression levels between samples and match adjacent non-tumor samples | [36, 82] |
| AC026166.2-001 & RP11-169D4.1-001 | – Down-regulated in LSCC samples and metastatic cervical lymph nodes; low expression associated with poor prognosis                                                                                                                                 | [83] |
| GAS5       | – Down-regulated in HNSCC and correlated with poor prognosis<br>– Circulating GAS5 as a prediction factor of patients’ response to radical chemoradiotherapy                                                                 | [62, 35, 51] |
| Inc-JPH1-7 | – Significant associated with survival of both HPV+ and HPV– patients and advanced tumor stage                                                                                                                                 | [63] |
| LET        | – Down-regulated in NPC samples; correlated with clinical stage, tumor size and lymph node involvement; low expression correlated with poor RFS and OS                                                                 | [68] |
| IncRNA-ROR | – Over-expressed in NPC samples and influence on chemoresistance                                                                                                                                              | [72] |
| XIST       | – Over-expressed in NPC samples and NPC cancer cell lines; connected with poorer OS; an independent risk factor for prognosis                                                                                   | [84] |
| MEG3       | – Down-regulated in TSCC samples and cell lines; correlated with tumor size; low level associated with poorer OS<br>– Lack of differences in expression levels between OSCC samples and matched adjacent non-tumor samples | [36, 85] |
| MALAT1     | – Over-expressed in LSCC, OSCC and TSCC samples – especially in the case of metastatic TSCC correlated with cervical lymph node metastasis<br>– In OSCC low expression significantly increased patients’ OS<br>– Lack of differences in expression levels between OSCC samples and match adjacent non-tumor samples and no differences between metastatic and non-metastatic samples | [36, 55, 71, 86] |
| H19        | – Over-expressed in LSCC samples; correlated with the tumor grade, differentiation, neck nodal metastasis, clinical stage and poorer OS                                                                                                                                 | [87] |
| lincRNA NAG7 (LINC00312) | – Down-regulated in NPC samples; correlated with lymph node metastasis, clinical stage and tumor size; high expression of LINC00312 is associated with better DFS and OS in patients without lymph node metastasis, in patients with positive lymph nodes the higher expression of LINC00312 significantly connected with poor DFS and OS | [12] |
| NKILA      | – Down-regulated in TSCC samples; correlated with clinical parameters, metastasis and poor DFS and OS                                                                                                                                 | [88] |
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