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Altered microRNA processing proteins in HPV-induced cancers

Barbara C Snoek[1], Iris Babion[1], Danijela Koppers-Lalic[2], Dirk M Pegtel[1] and Renske DM Steenbergen[1]

High-risk human papilloma virus (hrHPV) infections are associated with the development of anogenital cancers, in particular cervical cancer, and a subset of head and neck cancers. Previous studies have shown that microRNAs (miRNAs) contribute to the development and progression of HPV-induced malignancies. miRNAs are small non-coding RNAs that exist as multiple length and sequence variants, termed isomiRs. Efficient processing of miRNAs and generation of isomiRs is accomplished by several processing proteins. Deregulation of Drosha, AGO2, and TENT2, among others, has been observed in HPV-induced cancers and was even found at the precancerous stage. This suggests that miRNA processing proteins may be involved during early cancer development and that the generated isomiRs could provide promising biomarkers for early cancer diagnosis.

Addresses
[1] Amsterdam UMC, Vrije Universiteit Amsterdam, Pathology, Cancer Center Amsterdam, De Boelelaan 1117, Amsterdam, Netherlands
[2] Amsterdam UMC, Vrije Universiteit Amsterdam, Neurosurgery, Cancer Center Amsterdam, De Boelelaan 1117, Amsterdam, Netherlands

Corresponding author: Steenbergen, Renske DM (r.steenbergen@vumc.nl)

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Introduction

Human papilloma viruses (HPVs) belong to a family of small non-enveloped viruses containing double-stranded circular DNA genomes that can be categorized into mucosal or cutaneous HPV types, depending on their preferential site (mucosa or skin) of infection [1]. Mucosal HPV types are classified as low-risk and high-risk based on their potential to induce malignant transformation in infected cells. Low-risk HPV types, such as HPV6 and HPV11, represent the most prevalent HPV types and can cause benign warts without inducing malignancy. High-risk HPV types, such as HPV16 and 18, are a necessary cause for the development of most cervical cancers [2] and are responsible for a large proportion of other anogenital cancers, such as anal, penile, vaginal, and vulvar cancer [3]. Moreover, HPV is the causative agent of a substantial and rising subset of head and neck squamous cell carcinomas (HNCSCC). World-wide estimates indicate that approximately 22% of tumors arising in the oropharynx (OPSCC) are attributable to HPV, with highest frequencies found in tonsillar squamous cell carcinomas (47%) [4,5].

MicroRNAs (miRNAs) play pivotal roles in post-transcriptional regulation of gene expression and are often deregulated in cancer, including HPV-induced cancers [6•,7]. These small non-coding RNAs (~22 nucleotides) are expressed by almost all eukaryotes, and even by some viruses [8]. In cancer, miRNAs can either function as tumor suppressors or oncogenes (oncomiRs) depending on the genes and pathways they target [7].

The biogenesis of miRNAs is a tightly regulated multistep process accomplished by several miRNA processing proteins (Table 1 and Figure 1). Most miRNA genes are transcribed by RNA polymerase II in the nucleus [9•]. Approximately half of all known miRNA genes reside in host genes, the majority being processed from introns of protein-coding genes. miRNAs are encoded as individual genes (monocistronic) or in miRNA clusters that can contain a few to several hundred different miRNAs [10]. These so-called polycistronic miRNA clusters are transcribed together, but processed as individual miRNAs. Following transcription, the primary miRNA (pri-miRNA) is cleaved by the microprocessor; a nuclear protein complex that comprises the RNase III enzyme Drosha and its cofactor the double-stranded RNA (dsRNA)-binding protein (dsRBP) DiGeorge critical region 8 (DGCR8) [9•].

Cleavage of the pri-miRNA by the microprocessor releases a ~60–70 nucleotide hairpin-shaped precursor miRNA (pre-miRNA) with an overhang at the 3' end of either 2 nucleotides (group I miRNAs) or 1 nucleotide (group II miRNAs). The pre-miRNA is subsequently transported from the nucleus to the cytoplasm via the export receptor exportin 5 (XPO5), which creates a transport complex with RAN*GTP. In the cytoplasm, the RNase III enzyme Dicer cooperates with *trans*-activation-responsive RNA-binding protein (TRBP, also known as TARBP2) and cleaves the pre-miRNA near

Table 1

Proteins involved in miRNA and isomiR biogenesis

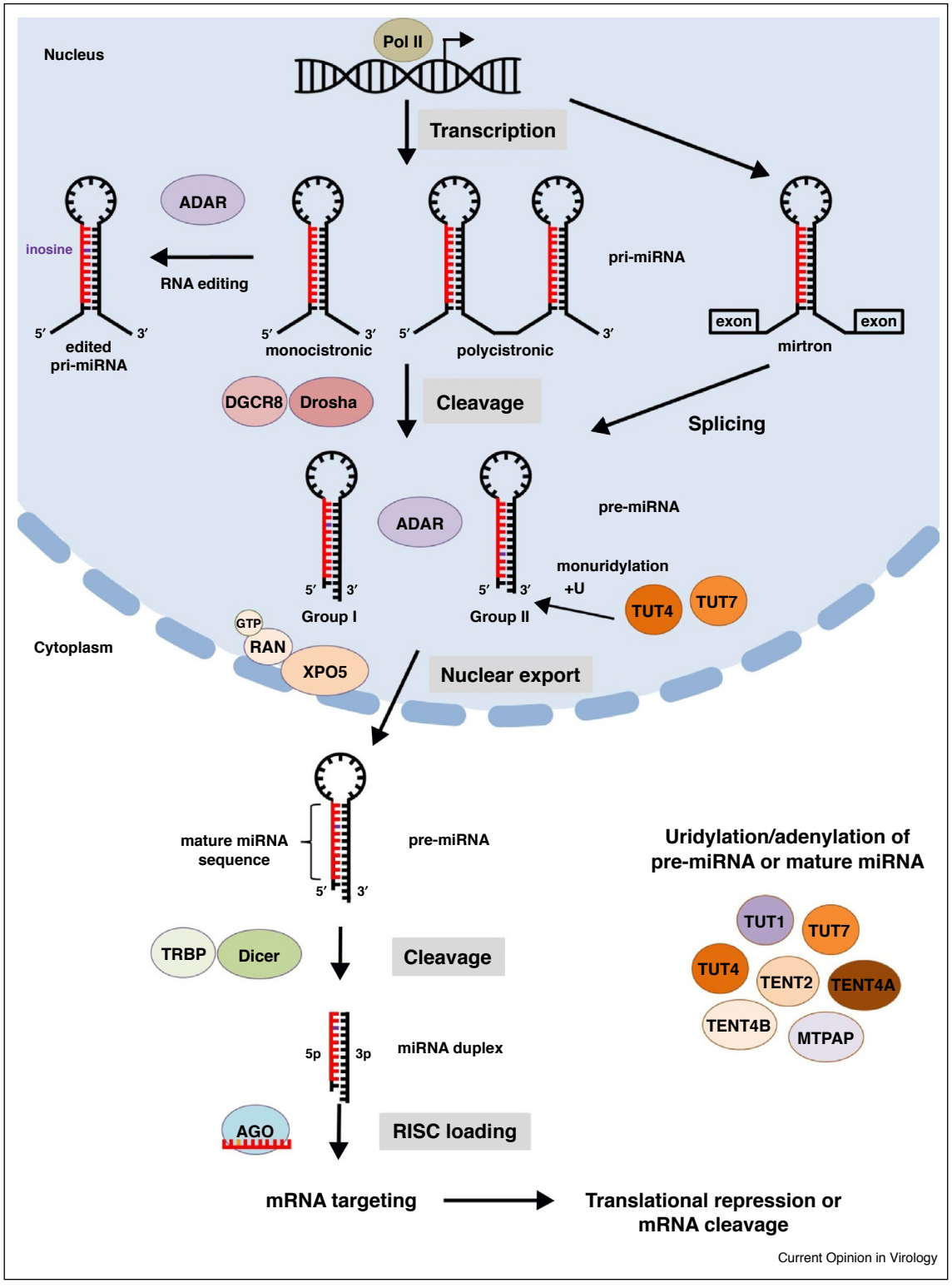
Name	Synonyms	Type	Function/activity	Altered in HPV + cancers	Refs.
miRNA biogenesis					
Drosha	RNASEN	RNase III endonuclease	Cleavage of pri-miRNA	Up	[26,28•,29,30], in-house data
DGCR8	C22orf12	Double-stranded RNA binding protein	Non-catalytic subunit of the microprocessor complex	Up	[37••]
XPO5		Nuclear export protein	Transport of pre-miRNA	Up	In-house data
Dicer	MNG1	RNase III endonuclease	Cleavage of pre-miRNA	Up/down	[29,30,35,36,37••]
AGO2	EIF2C2, CASC7	Endonuclease	Catalytic component of RISC	Up	[43,45], in-house data
TRBP	TARBP2	RNA binding protein	Required for efficient Dicer processing	Unknown	
IsomiR biogenesis					
ADAR		Adenosine deaminase	Adenosine to inosine editing	Up	[50,51•], in-house data
MTPAP	TENT6, PAPD1	Mitochondrial poly (A) polymerase	3' Adenylation	Unknown	
TENT2	PAPD4, GLD2	Cytoplasmic poly (A) polymerase	3' Adenylation	Up	In-house data
TENT4A	PAPD7, POLS	Nuclear poly (A) polymerase	3' Adenylation	Up	In-house data
TENT4B	PAPD5	Cytoplasmic poly (A) polymerase	3' Adenylation	Unknown	
TUT1	TENT1, RBM21, Star-PAP	Nuclear poly (A) polymerase; terminal uridyltransferase	3' Adenylation and uridylation	Unknown	
TUT4	ZCCHC11, TENT3A	Terminal uridyltransferase	3' Uridylation	Unknown	
TUT7	ZCCHC6, TENT3B	Terminal uridyltransferase	3' Uridylation	Down	In-house data

the terminal loop, generating a 20–25 nucleotide long miRNA duplex. The name of the miRNA depends on the direction from which the miRNA originates; the 5p strand arises from the 5' end while the 3p arm comes from the 3' end. One strand of the mature miRNA duplex, the so-called guide strand, assembles into the RNA-induced silencing complex (RISC), where it directly interacts with a member of the Argonaute protein family (AGO), while the so-called passenger strands gets discarded. The classic model predicts that the mature miRNA bound by AGO directs the complex to complementary target mRNAs for transcriptional gene silencing [11]. While most miRNA binding sites are located in the 3' untranslated region (3′UTR) of the target mRNA, other binding sites, that is, the 5′UTR and the coding sequence, have also been described [12]. The so-called seed sequence of the miRNA — a nucleotide region located at the 5' end of the miRNA (nucleotides 2–8) — is essential for and guides the binding of miRNA to mRNA [13]. Upon binding of RISC, the targeted mRNA can be post-transcriptionally silenced by various mechanisms, depending on the type of AGO protein and the cellular context. The most prevalent gene silencing mechanism by RISC is translational repression which can be accomplished by inhibition of translational initiation or by target mRNA deadenylation and degradation [14]. Another well-known RISC mechanism is mRNA cleavage. The reading frame of the targeted mRNA is cleaved and degraded by exonucleases. In humans four AGO proteins are known, of which AGO2 is the only AGO protein capable of cleaving targeted mRNAs. In a 2017 study on herpesviruses evidence was provided that miRNAs target mRNAs via binding to virus-encoded small non-coding RNAs [15]. The miRNA in this scenario seems to recruit Argonaute proteins to target mRNAs which is necessary for mRNA repression. With these various RISC mechanisms and the potential of miRNAs to target hundreds of RNA transcripts, individual miRNAs can fine-tune the expression of many genes. The above described miRNA biogenesis pathway is considered the canonical pathway. However, not all miRNAs are processed via the canonical route and several non-canonical miRNA biogenesis pathways have been described, including Drosha-independent and Dicer-independent pathways [9•]. The most well-known non-canonical type of miRNAs are the 'mirtrons'.

Mirtrons originate from introns that bypass Drosha cleavage and, upon splicing, function as pre-miRNAs that can be exported to the cytoplasm by XPO5 and subsequently processed by Dicer for loading into RISC (Figure 1).

In addition to the aforementioned miRNA processing machinery, several other proteins exist that can influence miRNA processing. Recent advances in small RNA sequencing technology have revealed that a single miRNA can generate numerous length and sequence variants during miRNA processing [16,17]. These miRNA variants are termed isomiRs and can encompass substitutions, insertions or deletions, 5' and/or 3' cleavage site variations, and terminal non-templated additions (NTA) [18]. Variations at the cleavage site arise from changes in Drosha and Dicer processing and substitutions by RNA editing enzymes, while terminal NTAs are generated by nucleotidyl transferases (NTases).

Figure 1



The biogenesis of miRNAs.
Transcription of primary miRNA (pri-miRNA) by RNA polymerase II is followed by cleavage of the pri-miRNA by the microprocessor complex Drosha–DGCR8 in the nucleus. The resulting precursor miRNA (pre-miRNA) is exported from the nucleus to the cytoplasm by XPO5 where it is further cleaved by Dicer in complex with TRBP. One strand of the mature miRNA duplex, together with an AGO protein, is incorporated in RISC and subsequently targets mRNAs for degradation or translational repression.

Alterations in any of the proteins involved in miRNA processing might disturb efficient maturation of precursor miRNAs and/or affect isomiR biogenesis, and could drastically alter miRNA function.

At present, the importance of miRNAs in cancer is well established. By regulating the expression of both oncogenes and tumor suppressor genes they play vital roles in tumorigenesis [7]. MiRNA expression is both cell-type and tissue-type-specific and miRNAs can function either cell autonomously or in a non-cell-autonomous fashion [19]. Their deregulation is often observed in cancer and they serve as powerful biomarkers for cancer diagnosis and prognosis. In addition, a recent study revealed that isomiR profiles can discriminate among 32 cancer types [20••], suggesting that isomiRs provide a novel class of biomarkers. Also, the presence of particular isomiRs might be indicative of an upstream miRNA processing defect, such as genomic mutations, altered expression or changes in the localization of proteins involved in miRNA maturation and isomiR generation.

Interestingly, viruses have been shown to affect miRNA processing through their viral proteins in order to regulate cellular gene expression [21]. In HPV-induced cancers, many miRNAs have been found to be deregulated, as reviewed by Satapathy *et al.* [6•], Santos *et al.* [22], and Emmett *et al.* [23]. Furthermore, our recent small RNA sequencing analysis on HPV-positive cervical samples indicated that the isomiR distribution is also disturbed in HPV-induced cancers [24]. This finding, together with the notion that viruses can alter miRNA processes led us to review the current literature on miRNA processing proteins and their alterations during HPV-induced carcinogenesis to obtain further insight in the mechanisms driving miRNA deregulation.

We focus on miRNA processing proteins involved in canonical miRNA processing as well as proteins involved in isomiR biogenesis and only include studies in the context of HPV-mediated transformation. Hence, functional studies performed solely with HeLa cells, high-risk HPV18-positive cervical cancer cells, are not addressed in this review.

Proteins involved in canonical miRNA processing

Although numerous miRNAs with oncogenic function have been identified, several studies have shown that miRNA expression is globally suppressed in cancer compared to normal cells. This suggests that the miRNA processing machinery might be deregulated in cancer. In fact, HPV-induced cancers often show deregulated expression of Drosha and DGCR8 – key components of the microprocessor.

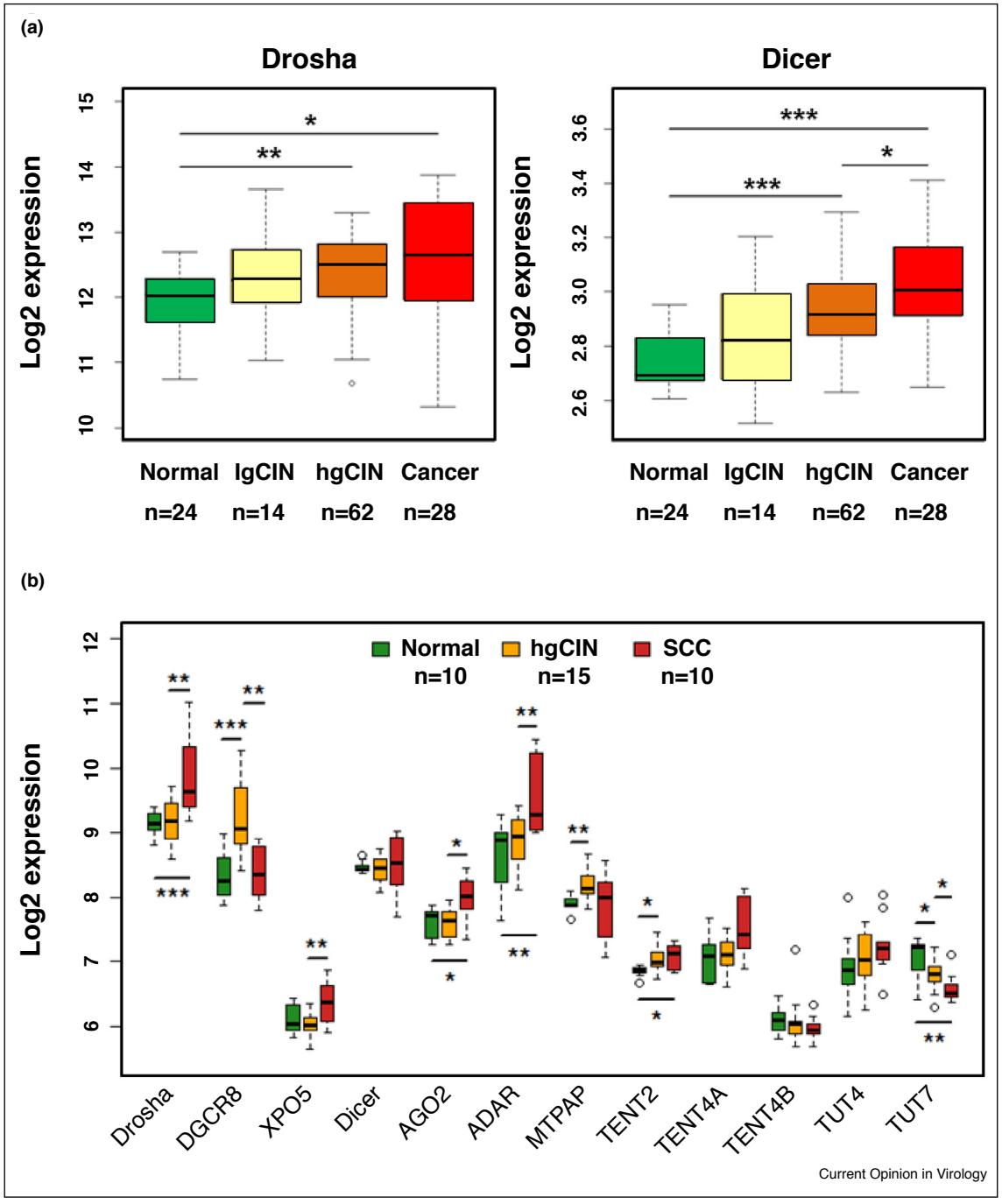
Drosha is located at chromosome 5p, which is one of the frequently gained chromosomal regions in cervical cancers [25,26]. It was found that all cervical cancers with 5p gain exhibited Drosha overexpression, indicating that upregulation of Drosha is a copy number driven event [25,27]. Harden and Munger recently showed that HPV-positive cervical cancer cell lines exhibit higher endogenous levels of Drosha than HPV-negative cervical cancer cells [28•]. They further demonstrated that co-expression of HPV16 viral oncogenes E6 and E7, and to a lesser extent expression of the individual genes, can cause overexpression of Drosha in both HPV-negative cervical cancer cells and primary human epithelial cells. Hence, both a gain of chromosome 5p and expression of the two viral oncoproteins E6 and E7 are likely to be involved in the upregulation of Drosha in cervical cancers. These data and the finding that a gain of 5p is also observed in 5–8% of high-grade cervical precancerous lesions [26] suggest that Drosha upregulation may already occur at the precancerous stage. Indeed, analysis of publicly available and in-house mRNA expression profiles confirmed an upregulation of Drosha expression in a subset of high-grade cervical precancerous lesions and most cervical cancers (Figure 2a and b) [29]. Enhanced expression of Drosha was found to be associated with altered cancer-associated miRNA profiles and enhanced cell motility and invasiveness [27,30]. These data support a role of Drosha as oncogenic driver in cervical cancer.

Furthermore, a recent study by De Ruyck *et al.* investigated whether genetic polymorphisms in miRNA target genes and processing genes including Drosha and **XPO5** could provide biomarkers for prognosis in HPV-positive and HPV-negative OPSCC patients [31]. Although HPV presence and other genetic polymorphisms were linked to prognosis, Drosha and XPO5 polymorphisms were not significantly associated with OPSCC outcome.

Another key component of the microprocessor, **DGCR8,** was demonstrated to be upregulated in cervical cancers compared to adjacent normal tissues [32]. Furthermore, the viral oncoprotein E7 was found to upregulate the expression of DGCR8. The elevated levels of DGCR8 subsequently resulted in increased expression of cancer-associated miR-27b, thereby initiating a cascade of events resulting

In addition to canonically processed miRNAs, mirtrons originate from introns that, upon splicing, function as pre-miRNAs. IsomiRs are miRNA variants that differ in their length or sequence makeup and can be generated by imprecise cleavage of Drosha and Dicer, nucleotide addition or removal, and RNA editing. Enzymes that are involved in isomiR biogenesis include the RNA editing enzyme ADAR and terminal nucleotidyl transferases (NTases) MTPAP, TENT2, TENT4A, TENT4B, TUT1, TUT4, and TUT7 that can catalyze the addition of either adenine (MTPAP, TENT2, TENT4A, TENT4B) or uridine (TUT4 and TUT7), or both (TUT1) (Table 1).

Figure 2



Expression levels of miRNA processing proteins in cervical tissues.
Log2 expression levels obtained from **(a)** publicly available data of Drosha and Dicer in cervical tissues (24 normal, 14 low-grade cervical intraepithelial neoplasia (lgCIN), 62 high-grade CIN (hgCIN), 28 cervical cancers), and **(b)** in-house mRNA microarray data of Drosha, DGCR8, XPO5, Dicer, AGO2, ADAR, MTPAP, TENT2, TENT4A, TENT4B, TUT4, and TUT7 in cervical tissues (10 normal, 15 hgCIN, and 10 SCC). Of note, the y-axis is differentially scaled for each plot. The Kruskall–Wallis omnibus test and the post-hoc non-parametric test were employed to compare expression levels between biological groups. Obtained P values were adjusted for multiple comparisons. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

in enhanced proliferation and reduced apoptosis. These findings suggest that DGCR8 is a cancer-promoting factor induced by HPV.

One of the most crucial players in miRNA maturation, **Dicer**, shows a heterogeneous expression pattern in cervical cancers. He and colleagues revealed that from

a set of 90 cervical squamous cell carcinomas (SCC), the majority (63.3%) showed high Dicer mRNA expression compared to adjacent normal cervical epithelium [33]. This expression pattern did not completely reflect Dicer protein levels, since only 49% of SCCs exhibited high Dicer protein expression, indicating that Dicer potentially undergoes post-transcriptional or post-translational regulation. Furthermore, low Dicer mRNA levels were associated with poor disease-free survival, which suggests that Dicer levels could be used as potential prognostic factor in cervical cancer. Conversely, Zhao *et al.* [34] and Lu *et al.* [35], demonstrated an overall downregulation of Dicer mRNA in the majority of cervical cancer tissues compared to normal tissues, but expression levels were rather variable. This discrepancy between the still limited number of studies may in part relate to differences in histotype and HPV type distributions. Our analysis on publicly available and in-house available mRNA data confirms a heterogeneous expression pattern of Dicer in cervical cancers (Figure 2a and b) [29].

Harden and Munger showed significantly higher Dicer mRNA levels in HPV16-positive cervical cancer cells compared to HPV-negative cervical cancer cells [28•], indicating that Dicer regulation may be HPV-dependent. A potential direct role of HPV16 E6 and E7 in Dicer upregulation is supported by the observed upregulation of Dicer upon ectopic expression of HPV16 E6/E7 in primary human epithelial cells. In contrast, HPV16 E6 has also been demonstrated to negatively regulate the expression of Dicer by inducing degradation of the transcription factor TAp63β, which is involved in Dicer activation [35].

Overexpression of the tumor suppressive miRNA miR-375 was able to restore TAp63β levels and miR-375 and Dicer expression were found to be positively correlated. Our previous data show that miR-375 expression is higher in adenocarcinomas than SCC [36] and The Cancer Genome Atlas (TCGA) data show that miR-375 levels are higher in HPV18-positive SCC compared to HPV16-positive SCC [37••]. Together, this suggests that Dicer levels are higher in HPV18-positive cancers and/or adenocarcinomas. However, studies on Dicer expression in HPV18-positive adenocarcinoma cell lines versus HPV16-positive SCC cell lines report varying findings [28•,35]. Furthermore, studies on single nucleotide polymorphisms (SNPs) in the Dicer gene showed reduced risk of cervical precancerous lesions [35], but no association with cervical cancer risk [38].

Although TRBP plays an important role in miRNA maturation, studies investigating how viral infections or malignant transformation may affect the function of TRBP are lacking.

Another important player in miRNA maturation and major component of RISC, **AGO2**, was found to exhibit higher mRNA levels in cervical cancer tissues than in the respective adjacent non-cancerous tissues [39]. Besides changes in its expression, AGO2 is a target for regulatory post-translational modifications. Phosphorylation of AGO2 was recently shown to strongly affect mRNA binding while no effect was observed on its capacity to bind miRNAs [40]. While this post-translational modification has been investigated in HeLa cells (HPV18-positive cervical cancer cells), it remains to be determined whether this phenomenon is involved in HPV-induced carcinogenesis.

For many years, Argonaute proteins have been viewed as cytoplasmic proteins. Recent studies, however, have revealed that RNA factors including Argonaute proteins can also be found in cell nuclei [41,42]. Although the exact functions of nuclear AGO2 are not completely understood, it has been suggested that nuclear AGO2 is involved in DNA repair among others [43]. Interestingly, AGO2 was found to be mainly located in the nucleus of primary human foreskin keratinocytes while cervical cancer cells exhibited mostly cytoplasmic AGO2 expression and only a minor expression in their nuclei [43]. Infection of primary keratinocytes with HPV16 or HPV18 did not influence AGO2 nuclear distribution, both when grown in monolayers and in organotypic cultures. Similar to primary keratinocytes, also in normal cervical epithelium AGO2 was primarily found in the nucleus [43]. However, in contrast to cervical cancer cell lines, AGO2 mainly displays as a nuclear protein in cervical cancer tissues [43]. This indicates that the subcellular distribution of AGO2 can be cell type-dependent and tissue context-dependent, and may change with cancer development.

Proteins involved in isomiR biogenesis

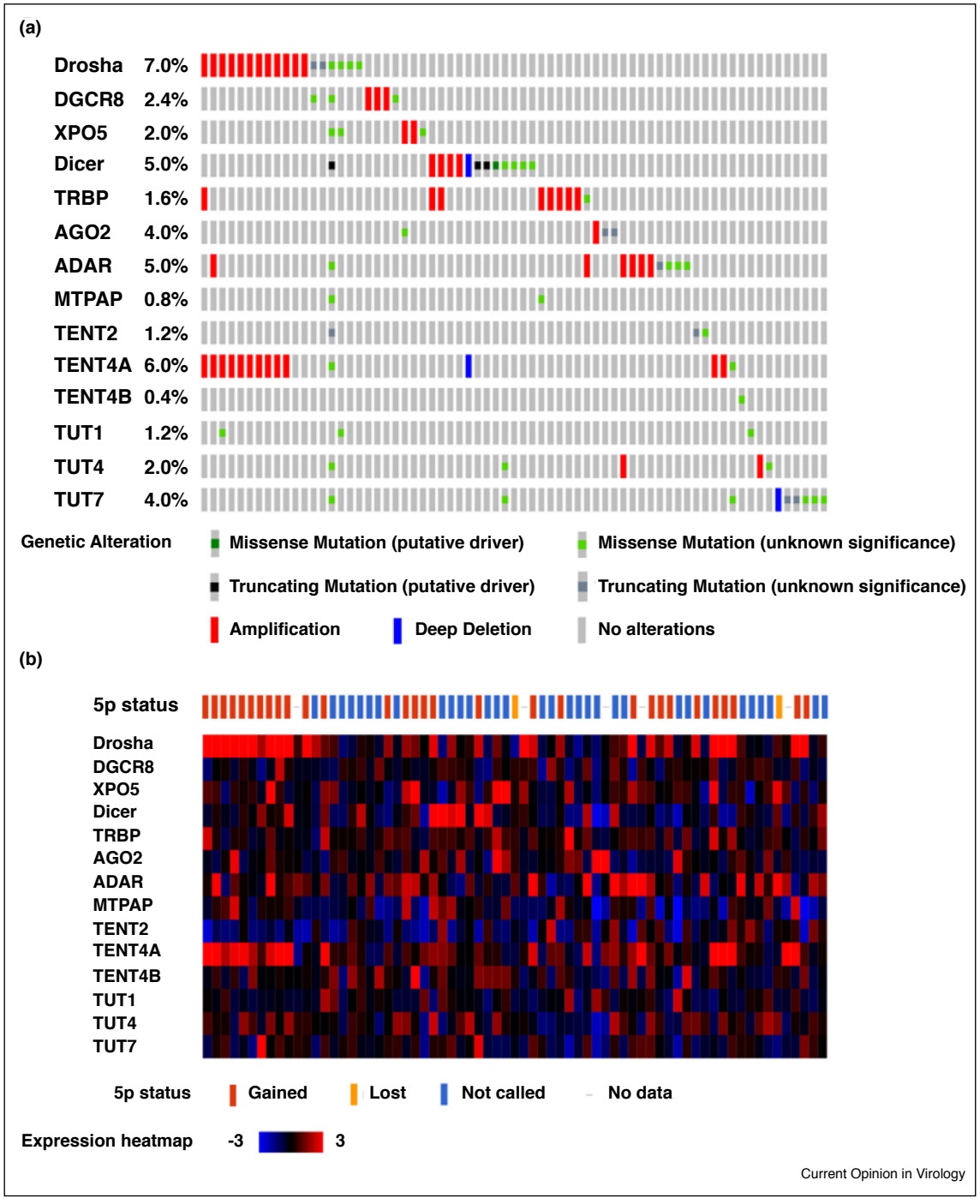
Besides their function in miRNA biogenesis, some of the above-mentioned miRNA processing proteins are also involved in the generation of miRNA variants, so-called isomiRs. IsomiRs originate from a single miRNA locus but can differ in their length and sequence makeup [16]. Imprecise cleavage of Drosha or Dicer can result in length differences at both the 3' and 5' end of the sequence. In this case, the isomiR sequence matches those of the miRNA gene but differs in length. These isomiRs are referred to as 'templated'. Terminal end sequence differences that do not match the miRNA gene are called 'non-templated'. Non-templated isomiRs can arise from (1) post-transcriptional addition of one or more nucleotides at the 5' or 3' end by NTases, or (2) RNA-editing enzymes that alter internal nucleotides. The latter are referred to as polymorphic isomiRs.

Polymorphic isomiRs are generated by RNA-editing enzymes such as adenosine deaminase acting on RNA (**ADAR**) [44]. ADARs convert adenosine (A) to inosine (I) in double-stranded RNAs. Primary miRNAs as well as

precursor and mature miRNAs are known to undergo A-to-I editing, which affects miRNA maturation and activity [45,46•]. For example, pri-miR-142 is highly edited by ADAR1 and/or ADAR2 and this editing results in inhibition of Drosha cleavage and thereby reduces the expression of miR-142 [47]. Also, A-to-I editing of miR-376 was shown to occur in the seed region of miR-376, altering its target profile [48].

Despite their importance in miRNA processing and function, only a few studies on ADAR in HPV-induced cancers have been reported. ADAR1 mRNA was found to be highly

Figure 3



Alterations of miRNA and isomiR processing proteins in samples from a TCGA study on 243 cervical SCCs.
(a) Oncoprint of genomic alterations and **(b)** 5p status and mRNA expression heatmap [37••].

expressed in cervical cancer tissues, while relatively low levels of ADAR1 in precancerous lesions and no expression in normal cervix were observed [49]. Moreover, cervical cancer patients that exhibited high ADAR1 expression had a lower overall survival compared to patients with low ADAR1 levels. Similar to those findings, results from a different study showed that mRNA levels of ADAR1 as well as ADAR2 are higher in cervical cancer tissues compared to the respective adjacent non-cancerous tissue [50]. These results indicate that ADARs are potentially involved in cervical carcinogenesis.

In recent years, several enzymes have been linked to isomiR biogenesis. These enzymes include terminal NTases MTPAP, TENT2 (PAPD4), TENT4A (PAPD7), TENT4B (PAPD5), TUT1, TUT4 (ZCCHC11), and TUT7 (ZCCHC6) [51•]. Most enzymes have multiple gene names, which can create some confusion as to their precise functioning (Table 1). In this article, we will use their HUGO-approved nomenclature, as was recently proposed by Menezes *et al.* [52•].

NTases are template-independent polymerases that add ribonucleotides to the end of RNA molecules [53]. They can possess either adenylation (addition of an adenine) or uridylation (addition of a uridine) activity, or both. Uridylation of miRNAs does not only create isomiRs but has also been shown to regulate miRNA biogenesis [52•]. Most pre-miRNAs have a 2 nucleotide 3' overhang upon Drosha cleavage that is recognized in the cytoplasm by Dicer (group I). However, some pre-miRNAs contain a single uridine at their 3'end (group II). These group II miRNAs include most let-7 family members as well as miR-105. Monouridylation — addition of one uridine — of these pre-miRNAs is needed in order to establish efficient Dicer processing and has been reported to be accomplished by TUT4 and TUT7 [54]. In addition to monouridylation, polyuridylation — addition of multiple uridines — is also involved in miRNA biogenesis. TUT4 was shown to add a polyU-tail to pre-let-7 which blocks Dicer cleavage [55]. The polyU-tail of pre-let-7 was subsequently recognized by exonuclease Dis3L2 and resulted in pre-let-7 degradation [56].

At present the role of NTases in cancer remains largely unexplored. To the best of our knowledge, only one study reports on NTases in HPV-induced cancers. In a study on factors that regulate E6 expression in cervical carcinogenesis it was found that the NTase **TUT1** (referred to as star-PAP) controls E6 polyadenylation and thereby its expression in cervical cancer cells [57]. Moreover, when TUT1 expression was reduced, cervical cancer cells became more sensitive to chemotherapeutic treatment. This indicates that TUT1 could serve as a potential drug target for the treatment of cervical cancer.

Since data on proteins involved in isomiR biogenesis in cervical cancer are limited, we used in-house available microarray mRNA profiles of cervical tissues to analyze their expression pattern in normal cervical epithelium, high-grade precancerous lesions (referred to as cervical intraepithelial neoplasia (CIN)), and cervical SCC. We observed significantly higher expression levels of Drosha, XPO5, AGO2, ADAR, and TENT2 in SCC compared to high-grade CIN and/or normal cervical tissues (Figure 2b). The expression of TUT7 was significantly reduced with severity of cervical disease. For Drosha and Dicer, a similar expression pattern was observed in a publicly available dataset of cervical tissues consisting of normal, low-grade and high-grade CIN, and cervical cancer (Figure 2a) [29].

Additionally we analyzed The Cancer Genome Atlas (TCGA) database (243 SCCs) [37••] for genomic and expression alterations of miRNA processing proteins in cervical cancer. Most genomic alterations are found for Drosha (7%) and TENT4A (6%) and mainly include amplifications (Figure 3a). Both genes are located on chromosome 5p, which is gained in many cervical cancers. Consistently, a gain of 5p is often accompanied by increased mRNA levels of Drosha and TENT4A (Figure 3b). Additionally, putative driver mutations were seen in Dicer ($n = 3$; Figure 3a). In general, genomic alterations seem to coincide with altered mRNA expression levels, which are prominently seen for Drosha and TENT4A, but also for Dicer and ADAR1 (Figure 3a and b). Moreover, TCGA data show that SCCs harboring a 5p gain exhibit increased Drosha mRNA levels (Figure 3b), which is in line with previous findings [25,27].

Conclusions

The above summarized findings indicate that miRNA processing proteins Drosha, DGCR8, Dicer, and AGO2 are deregulated in HPV-induced cancers. Furthermore, the viral oncoproteins E6 and E7 have been implicated in Drosha, DGCR8, and Dicer regulation, but the exact role of HPV on miRNA processing proteins needs to be further elucidated.

Despite a lack of studies focusing on NTases involved in isomiR generation in respect to HPV-induced cancers, data from TCGA and in-house studies indicate that several NTases such as TENT2 and TENT4A are altered in cervical cancer or even at the precancerous stage. This indicates that NTases are likely to be involved during the early steps of cancer development. Since alterations of NTase levels directly affect isomiR biogenesis, the generated isomiRs could provide promising biomarkers for early cancer diagnosis. Future studies are needed to elucidate the exact role of NTases during carcinogenesis and their modulation might provide a potential therapeutic avenue.

Conflict of interest statement

D.M. Pegtel is CSO of ExBiome B.V. and serves occasionally as advisor for Takeda. D. Koppers-Lalic is the

head of diagnostic research of ExBiome B.V. and R.D.M. Steenbergen has a minority share in Self-screen B.V.

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References and recommended reading

Papers of particular interest, published within the period of review, have been highlighted as:

- • of special interest
- •• of outstanding interest

1. Munger K, Baldwin A, Edwards KM, Hayakawa H, Nguyen CL, Owens M, Grace M, Huh K: **Mechanisms of human papillomavirus-induced oncogenesis**. *J Virol* 2004, **78**:11451-11460.

2. Crosbie EJ, Einstein MH, Franceschi S, Kitchener HC: **Human papillomavirus and cervical cancer**. *Lancet* 2013, **382**:889-899.

3. Bansal A, Singh MP, Rai B: **Human papillomavirus-associated cancers: a growing global problem**. *Int J Appl Basic Med Res* 2016, **6**:84-89.

4. Castellsagué X, Alemany L, Quer M, Halec G, Quirós B, Tous S, Clavero O, Alòs L, Biegner T, Szafarowski T *et al.*: **HPV Involvement in head and neck cancers: comprehensive assessment of biomarkers in 3680 patients**. *J Natl Cancer Inst* 2016, **108** djv403.

5. Leemans CR, Snijders PJF, Brakenhoff RH: **The molecular landscape of head and neck cancer**. *Nat Rev Cancer* 2018, **18**:269-282.

6. • Satapathy S, Batra J, Jeet V, Thompson EW, Punyadeera C: **MicroRNAs in HPV associated cancers: small players with big consequences**. *Expert Rev Mol Diagn* 2017, **17**:711-722.
An extensive review on dysregulated microRNAs in HPV associated cancers that discusses both intrasomal and exosomal miRNAs, the effect of HPV oncoproteins on miRNA expression, their functional involvement in HPV associated cancers and their potential as therapeutic targets.

7. Lin S, Gregory RI: **MicroRNA biogenesis pathways in cancer**. *Nat Rev Cancer* 2015, **15**:321-333.

8. Bruscella P, Bottini S, Baudesson C, Pawlotsky J-M, Feray C, Trabucchi M: **Viruses and miRNAs: more friends than Foes**. *Front Microbiol* 2017, **8**:824.

9. • Treiber T, Treiber N, Meister G: **Regulation of microRNA biogenesis and its crosstalk with other cellular pathways**. *Nat Rev Mol Cell Biol* 2019, **20**:5-20.
A recent review on microRNA biogenesis that summarizes the current knowledge of both canonical and non-canonical miRNA biogeneses and discusses novel developments.

10. Truscott M, Islam ABMMK, Frolov MV: **Novel regulation and functional interaction of polycistronic miRNAs**. *RNA* 2016, **22**:129-138.

11. Jonas S, Izaurralde E: **Towards a molecular understanding of microRNA-mediated gene silencing**. *Nat Rev Genet* 2015, **16**:421-433.

12. Moretti F, Thermann R, Hentze MW: **Mechanism of translational regulation by miR-2 from sites in the 5' untranslated region or the open reading frame**. *RNA* 2010, **16**:2493-2502.

13. Bartel DP: **MicroRNAs: target recognition and regulatory functions**. *Cell* 2009, **136**:215-233.

14. Pratt AJ, MacRae IJ: **The RNA-induced silencing complex: a versatile gene-silencing machine**. *J Biol Chem* 2009, **284**:17897-17901.

15. Gorbea C, Mosbruger T, Cazalla D: **A viral Sm-class RNA base-pairs with mRNAs and recruits microRNAs to inhibit apoptosis**. *Nature* 2017, **550**:275-279.

16. Neilsen CT, Goodall GJ, Bracken CP: **IsomiRs—the overlooked repertoire in the dynamic microRNAome**. *Trends Genet* 2012, **28**:544-549.

17. Morin RD, O'Connor MD, Griffith M, Kuchenbauer F, Delaney A, Prabhu A-L, Zhao Y, McDonald H, Zeng T, Hirst M *et al.*: **Application of massively parallel sequencing to microRNA profiling and discovery in human embryonic stem cells**. *Genome Res* 2008, **18**:610-621.

18. Cloonan N, Wani S, Xu Q, Gu J, Lea K, Heater S, Barbacioru C, Steptoe AL, Martin HC, Nourbakhsh E *et al.*: **MicroRNAs and their isomiRs function cooperatively to target common biological pathways**. *Genome Biol* 2011, **12**:R126.

19. Suzuki HI, Katsura A, Matsuyama H, Miyazono K: **MicroRNA regulons in tumor microenvironment**. *Oncogene* 2015, **34**:3085-3094.

20. •• Telonis AG, Magee R, Loher P, Chervoneva I, Londin E, Rigoutsos I: **Knowledge about the presence or absence of miRNA isoforms (isomiRs) can successfully discriminate amongst 32 TCGA cancer types**. *Nucleic Acids Res* 2017, **45**:2973-2985.
In this study more than 10 000 tumor datasets from TCGA were evaluated. The authors demonstrate that isomiR profiles can most accurately distinguish among 32 TCGA cancers. The most discriminative isomiRs could provide novel cancer biomarkers.

21. Libri V, Miesen P, van Rij RP, Buck AH: **Regulation of microRNA biogenesis and turnover by animals and their viruses**. *Cell Mol Life Sci* 2013, **70**:3525-3544.

22. Santos J, Peixoto da Silva S, Costa N, Gil da Costa R, Medeiros R: **The role of MicroRNAs in the metastatic process of high-risk HPV-induced cancers**. *Cancers (Basel)* 2018, **10**:493.

23. Emmett S, Whiteman DC, Panizza BJ, Antonsson A: **An update on cellular MicroRNA expression in human papillomavirus-associated head and neck squamous cell carcinoma**. *Oncology* 2018, **95**:193-201.

24. Snoek BC, Verlaat W, Babion I, Novianti PW, van de Wiel MA, Wilting SM, van Trommel NE, Bleeker MCG, Massuger LFAG, Melchers WJG *et al.*: **Genome-wide microRNA analysis of HPV-positive self-samples yields novel triage markers for early detection of cervical cancer**. *Int J Cancer* 2019, **144**:372-379.

25. Scotto L, Narayan G, Nandula SV, Subramaniyam S, Kaufmann AM, Wright JD, Pothuri B, Mansukhani M, Schneider A, Arias-Pulido H *et al.*: **Integrative genomics analysis of chromosome 5p gain in cervical cancer reveals target over-expressed genes, including Drosha**. *Mol Cancer* 2008, **7**:58.

26. Thomas LK, Bermejo JL, Vinokurova S, Jensen K, Bierkens M, Steenbergen R, Bergmann M, von Knebel Doeberitz M, Reuschenbach M: **Chromosomal gains and losses in human papillomavirus-associated neoplasia of the lower genital tract – a systematic review and meta-analysis**. *Eur J Cancer* 2014, **50**:85-98.

27. Muraldihar B, Goldstein L, Ng G, Winder D, Palmer R, Gooding E, Barbosa-Morais N, Mukherjee G, Thorne N, Roberts I *et al.*: **Global microRNA profiles in cervical squamous cell carcinoma depend on Drosha expression levels**. *J Pathol* 2007, **212**:368-377.

28. • Harden ME, Munger K: **Perturbation of DROSHA and DICER expression by human papillomavirus 16 oncoproteins**. *Virology* 2017, **507**:192-198.
DROSHA is linked to a gain of chromosomal 5p. In this study the authors demonstrate that DROSHA expression is also regulated by high-risk HPV E6/E7. Besides DROSHA, DICER expression is also affected by HPV E6/E7.

29. den Boon JA, Pyeon D, Wang SS, Horswill M, Schiffman M, Sherman M, Zuna RE, Wang Z, Hewitt SM, Pearson R *et al.*: **Molecular transitions from papillomavirus infection to cervical precancer and cancer: role of stromal estrogen receptor signaling**. *Proc Natl Acad Sci U S A* 2015, **112**:E3255-E3264.

30. Muralidhar B, Winder D, Murray M, Palmer R, Barbosa-Morais N, Saini H, Roberts I, Pett M, Coleman N: **Functional evidence that Drosha overexpression in cervical squamous cell carcinoma affects cell phenotype and microRNA profiles**. *J Pathol* 2011, **224**:496-507.

31. De Ruyck K, Duprez F, Ferdinande L, Mbah C, Rios-Velazquez E, Hoebers F, Praet M, Deron P, Bonte K, Speel E-J *et al.*: **A let-7 microRNA polymorphism in the KRAS 3′-UTR is prognostic in oropharyngeal cancer**. *Cancer Epidemiol* 2014, **38**:591-598.

32. Liu F, Zhang S, Zhao Z, Mao X, Huang J, Wu Z, Zheng L, Wang Q: **MicroRNA-27b up-regulated by human papillomavirus 16 E7 promotes proliferation and suppresses apoptosis by targeting polo-like kinase2 in cervical cancer**. *Oncotarget* 2016, **7**:19666-19679.

33. He L, Wang H-Y, Zhang L, Huang L, Li J-D, Xiong Y, Zhang M-Y, Jia W-H, Yun J-P, Luo R-Z *et al.*: **Prognostic significance of low DICER expression regulated by miR-130a in cervical cancer**. *Cell Death Dis* 2014, **5**:e1205.

34. Zhao H, Jin X, Su H, Deng X, Fang Y, Shen L, Xie C: **Down-regulation of Dicer expression in cervical cancer tissues**. *Med Oncol* 2014, **31**:937.

35. Lu H, Qi Z, Lin L, Ma L, Li L, Zhang H, Feng L, Su Y: **The E6-TAp63β-Dicer feedback loop involves in miR-375 downregulation and epithelial-to-mesenchymal transition in HR-HPV+ cervical cancer cells**. *Tumor Biol* 2016, **37**:15805-15811.

36. Babion I, Snoek BC, Novianti PW, Jaspers A, van Trommel N, Heideman DAM, Meijer CJLM, Snijders PJF, Steenbergen RDM, Wilting SM: **Triage of high-risk HPV-positive women in population-based screening by miRNA expression analysis in cervical scrapes; a feasibility study**. *Clin Epigenet* 2018, **10**:76.

37. •• Network TCGAR: **Integrated genomic and molecular characterization of cervical cancer**. *Nature* 2017, **543**:378-384.
The most comprehensive data integration of molecular features in cervical cancers performed to date.

38. Chen J, Qin Z, Pan S, Jiang J, Liu L, Liu J, Chen X, Hu Z, Shen H: **Genetic variants in RAN, DICER and HIWI of microRNA biogenesis genes and risk of cervical carcinoma in a Chinese population**. *Chin J Cancer Res* 2013, **25**:565-571.

39. Guo J, Lv J, Liu M, Tang H: **miR-346 up-regulates Argonaute 2 (AGO2) protein expression to augment the activity of other MicroRNAs (miRNAs) and contributes to cervical cancer cell malignancy**. *J Biol Chem* 2015, **290**:30342-30350.

40. Quévillon Huberdeau M, Zeitler DM, Hauptmann J, Bruckmann A, Fressigné L, Danner J, Piquet S, Strieder N, Engelmann JC, Jannot G *et al.*: **Phosphorylation of Argonaute proteins affects mRNA binding and is essential for microRNA-guided gene silencing in vivo**. *EMBO J* 2017, **36**:2088-2106.

41. Castanotto D, Zhang X, Alluin J, Zhang X, Rüger J, Armstrong B, Rossi J, Riggs A, Stein CA: **A stress-induced response complex (SIRC) shuttles miRNAs, siRNAs, and oligonucleotides to the nucleus**. *Proc Natl Acad Sci U S A* 2018, **115**:E5756-E5765.

42. Zhang X, Shen B, Cui Y: **Ago HITS-CLIP expands microRNA-mRNA interactions in nucleus and cytoplasm of gastric cancer cells**. *BMC Cancer* 2019, **19**:29.

43. Sharma NR, Wang X, Majerciak V, Ajiro M, Kruhlak M, Meyers C, Zheng Z-M: **Cell type- and tissue context-dependent nuclear distribution of human Ago2**. *J Biol Chem* 2016, **291**:2302-2309.

44. Kawahara Y, Megraw M, Kreider E, Iizasa H, Valente L, Hatzigeorgiou AG, Nishikura K: **Frequency and fate of microRNA editing in human brain**. *Nucleic Acids Res* 2008, **36**:5270-5280.

45. Nishikura K: **Functions and regulation of RNA editing by ADAR deaminases**. *Annu Rev Biochem* 2010, **79**:321-349.

46. • Li L, Song Y, Shi X, Liu J, Xiong S, Chen W, Fu Q, Huang Z, Gu N, Zhang R: **The landscape of miRNA editing in animals and its impact on miRNA biogenesis and targeting**. *Genome Res* 2018, **28**:132-143.
By profiling more than 200 pri-miRNA and over 4000 mature miRNA samples, the authors present a quantitative atlas of miRNA A-to-I editing in human, mouse, and *Drosophila*.

47. Yang W, Chendrimada TP, Wang Q, Higuchi M, Seeburg PH, Shiekhattar R, Nishikura K: **Modulation of microRNA processing and expression through RNA editing by ADAR deaminases**. *Nat Struct Mol Biol* 2006, **13**:13-21.

48. Kawahara Y, Zinshteyn B, Sethupathy P, Iizasa H, Hatzigeorgiou AG, Nishikura K: **Redirection of silencing targets by adenosine-to-inosine editing of miRNAs**. *Science* 2007, **315**:1137-1140.

49. Chen Y, Wang H, Lin W, Shuai P: **ADAR1 overexpression is associated with cervical cancer progression and angiogenesis**. *Diagn Pathol* 2017, **12**:12.

50. Chen W, He W, Cai H, Hu B, Zheng C, Ke X, Xie L, Zheng Z, Wu X, Wang H: **A-to-I RNA editing of BLCAP lost the inhibition to STAT3 activation in cervical cancer**. *Oncotarget* 2017, **8**:39417-39429.

51. • Warkocki Z, Liudkovska V, Gewartowska O, Mroczek S, Dziembowski A: **Terminal nucleotidyl transferases (TENTs) in mammalian RNA metabolism**. *Philos Trans R Soc Lond B Biol Sci* 2018, **373**.
A review on terminal nucleotidyltransferases that summarizes the current knowledge about their functions and mechanisms of action.

52. • Menezes MR, Balzeau J, Hagan JP: **3' RNA uridylation in epitranscriptomics, gene regulation, and disease**. *Front Mol Biosci* 2018, **5**:61.
This review describes the functional roles and significance of 3'terminal RNA uridylation, discusses the responsible enzymes, and describes the diverse functions of 3'terminal uridylation in regulating both mRNAs and non-coding RNAs.

53. Martin G, Keller W: **RNA-specific ribonucleotidyl transferases**. *RNA* 2007, **13**:1834-1849.

54. Heo I, Ha M, Lim J, Yoon M-J, Park J-E, Kwon SC, Chang H, Kim VN: **Mono-uridylation of pre-microRNA as a key step in the biogenesis of group II let-7 microRNAs**. *Cell* 2012, **151**:521-532.

55. Heo I, Joo C, Kim Y-K, Ha M, Yoon M-J, Cho J, Yeom K-H, Han J, Kim VN: **TUT4 in concert with Lin28 suppresses microRNA biogenesis through pre-microRNA uridylation**. *Cell* 2009, **138**:696-708.

56. Chang H-M, Triboulet R, Thornton JE, Gregory RI: **A role for the Perlman syndrome exonuclease Dis3l2 in the Lin28-let-7 pathway**. *Nature* 2013, **497**:244-248.

57. Li W, Anderson RA: **Star-PAP controls HPV E6 regulation of p53 and sensitizes cells to VP-16**. *Oncogene* 2014, **33**:928-932.