Guanine content of microRNAs is associated with their tumor suppressive and oncogenic roles in lung and breast cancers

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Running Head

Precursor microRNA's guanine content in lung and breast cancers.

Contributions

(1) Conception and design: AC

(2) Administrative support: MAB-A

(3) Provision of study materials or patients: AC, MAB-A

(4) Collection and assembly of data: All authors

(5) Data analysis and interpretation: AC, YS

(6) Manuscript writing: All authors

(7) Final approval of manuscript: All authors.

Abstract

Background: microRNAs (miRNAs, miRs) are small noncoding RNAs that negatively regulate gene expression at the post-transcriptional level and fine-tune gene functions. A global repression of miRNAs expression in different types of human tumors, after exposure to cigarette-smoke, or to the hormone estrogen, have been shown to be associated with guanine (G) enrichment in the terminal loops (TLs) of their precursors.

Methods: we integrated the G content of miRNA mature forms and precursor miRNA TLs with their described function in the literature, using the PubMed database. Gene Ontology term analysis was used to describe the pathways in which the G-enriched miRNA targets are involved.

Results: herein we show an association between the relative G enrichment of precursor miRNAs TLs and their tendency to act as tumor suppressor miRs in human lung and breast cancers. Another association was observed between the high G content of the miRNAs 5-mature forms and their tendency to act as oncomiRs.

Conclusions: the results support previous findings showing that the G sequence content is an important feature determining miRNA expression and function, and open the way for future cancer investigations in this direction.

Keywords: Guanine, microRNA, Lung cancer, Breast cancer, Tumor suppressor, Oncogene.

Background

44 MicroRNAs (miRNAs) are endogenous ~22-nucleotides RNA molecules, that
45 negatively regulate gene expression at the post-transcriptional level and are
46 implicated in the pathogenesis of many human diseases, including cancer, where they
47 can act as tumor suppressive miRNAs (tumor suppressive miRs) or as oncogenic
48 miRNAs (oncomiRs) [1, 2]. Generally, oncomiRs are overexpressed in cancers while
49 tumor suppressive miRs are underexpressed, however, a comprehensive reduction in
50 miRNA was commonly observed in human cancers, where miRNAs showed lower
51 expression levels in tumors and cancer cell lines compared with normal tissues [3-6].
52 In addition, a widespread repression of miRNA expression has also been reported
53 after exposure to cigarette-smoke (CS) [7-9], treatment with the hormone estrogen
54 [10-12], and c-Myc activation [13]. These aforementioned alterations in miRNA
55 expression can occur as a result of affecting the transcription of miRNA genes [13],
56 miRNA export from the nucleus [14], or at any stage of the miRNA maturation
57 process by modulation of key regulators or components of the miRNA biogenesis
58 pathway, including Drosha and Dicer [15].
59 Findings suggest that the miRNA terminal loop (TL) is an important platform for
60 different RNA-binding proteins (RBPs) that act as activators or repressors of Drosha
61 and Dicer processing, and selectivity regulate miRNAs by binding to guanine (G)-
62 enriched motifs in the RNA TLs of their precursors [16]. It was shown that miRNAs
63 with the tetra-nucleotide sequence motif GGAG in their TLs were regulated through
64 binding of the RBP Lin28, which interferes with Dicer processing [17], and that the
65 sequence AGGGU in the TL mediates regulation of miRNA biogenesis by the KH-
66 type splicing regulatory protein (KSRP) RBP [18]. It was recently shown that
67 modification of KSRP resulted in the downregulation of a subset of TL-G-rich
68 miRNAs and promoted tumorigenesis [19].

69 Izzotti and Pulliero showed in their study that the G content of the TLs of
70 miRNAs, which are involved in stress response, is higher than the G content of the
71 other miRNAs [20]. We have recently found, using bioinformatic data analysis of
72 zebrafish, mouse, and human breast cancer cell line, an association between the
73 widespread miRNAs reduction that is observed after estrogen (17β-estradiol; E2)
74 exposure and a high TL-G content in their precursors [21]. In addition, we also
75 showed that similar G enrichment exists in TLs of downregulated miRNAs found in
76 different human cancers [22]. Here, we bioinformatically analyzed the sequences of
77 over 250 human miRNAs, and show the association between miRNAs G-content and
78 their known function as tumor suppressors and oncogenes in lung and breast cancers.

79 **Materials and Methods**

80 *Literature-data mining.*

81 Literature searches were performed in the PubMed literature database for original
82 articles written in the English language focusing on miRNAs and lung or breast
83 cancers. The searches included a specific miRNA term paired with the key words;
84 'lung cancer' or 'breast cancer'. No restriction was set for the publication date. Only
85 articles showing a role for miRNAs, by using functional studies, were selected. Where
86 applicable, the direct target genes of the investigated miRNAs were retrieved from the
87 above articles.

88 *Bioinformatic tools.*

89 All miRNA precursor sequences were obtained from the Sanger Institute miRBase
90 database version 21 (http://microrna.sanger.ac.uk/sequences/). DAVID v6 functional
91 annotation tool was used to identify enriched GO terms of genes.

92 *Nucleotide composition analysis.*

93 Calculation of nucleotide composition in miRNA precursors was determined using
94 the compseq algorithm (http://emboss.bioinformatics.nl/cgi-bin/emboss/compseq).
95 Input sequences included precursor miRNAs (pre-miRNAs) stem-loops (SLs), TLs, 5-
96 and 3-mature miRNAs of the tumor suppressive miRs and oncomiRs (miRNA lists
97 and their sequences are presented in Supplementary Table 1).

98 *Statistical data analysis.*

99 A one-way ANOVA, post-hoc Tukey HSD Test was performed for comparison
100 between G enrichment in SLs, TLs, and mature miRNAs of the tumor suppressive
101 miRs and oncomiRs. Statistical analysis was performed using the software XLSTAT
102 (Addinsoft Inc., Paris, France).

103 **Results**

104 Since miRNAs downregulation in cancer is associated with the relative G enrichment
105 of their TL sequences [22], we asked whether there is also a relation between the
106 relative G content of miRNAs and their known function in cancer. For this purpose,
107 255 human pre-miRNA sequences were retrieved from the miRBase database and
108 used for further analysis. Each sequence was divided into its different structural
109 constituents; the TL and the two mature miRNAs (5-mature and 3-mature forms). The
110 complete list of miRNA sequences is presented in Supplementary Table 1. For each
111 of these miRNAs the complete pre-miRNA, the TL, and the 5 and 3-mature miRNA
112 sequences were analyzed for evaluation of nucleotide composition (Supplementary
113 Table 1). Next, we filtered the miRNA list and selected those with relatively high G
114 content (more than 35%) or low G content (less than 15%) in their TL and 5-mature
115 sequences, and for the resulted 105 miRNAs, we searched for known functions in
116 lung and breast cancers, by mining publicly available data in the PubMed database.

117 The search resulted in 420 articles; 109 studied on oncomiRs and 311 on tumor
118 suppressive miRs.
119 The results show that when presenting the number of the articles respectively to
120 the G percentage of miRNAs TLs, tumor suppressive miRs are found to be more G-
121 enriched in their TLs, while oncomiRs show the opposite trend (Figure 1A). When
122 presenting the number of the articles respectively to the G percentage of 5-mature
123 miRNAs, the oncomiRs appear to be more G-enriched in their 5-mature forms (Figure
124 1B). From this list, we selected only those miRNAs that showed a tendency to act as
125 either tumor suppressive miRs or oncomiRs, in both lung and breast cancers. A list of
126 84 miRNAs was obtained and sub-grouped into oncomiRs (25 miRNAs) and tumor
127 suppressive miRs (59 miRNAs) (Supplementary Table 2).
128 The results show that the TLs of the tumor suppressive miRs group are more
129 enriched in G (1.33 fold) than the group of oncomiRs (Figure 2). No such enrichment
130 was observed when the same analysis was conducted with the complete pre-miRNA
131 SL sequences, or with the 5 and 3-mature miRNAs (Figure 2). Moreover, this
132 enrichment is even more prominent when analyzing the dual G (GG) content, where
133 the TLs of the tumor suppressive miRs group are more enriched in GG (2.1 fold) than
134 the group of oncomiRs (Figure 2). Also here, no such enrichment was observed when
135 the same analysis was conducted with the complete SLs, despite this time the GG
136 content of the 3-mature miRNAs was also slightly enriched (1.32 fold). Also in the
137 case of triple G (GGG), only the TLs of the tumor suppressive miRs group are more
138 enriched in G (1.34 fold) than the group of oncomiRs (Figure 2). Interestingly, the G
139 content of the 5-mature miRNAs has shown the opposite trend than the TLs, and was
140 slightly enriched in oncomiRs relative to tumor suppressive miRs (0.87 fold) (Figure
141 2), and this trend of enrichment in the 5-mature forms was even more pronounced

142 when looking at the GG (0.65 fold) and GGG (0.39 fold) content (Figure 2).
143 Statistical analysis revealed a significant difference between TLs and 5-mature
144 miRNAs G, GG and GGG content of the tumor suppressive miRs relative to
145 oncomiRs (one-way ANOVA, P=0.01). Together, the above results show an
146 association between high TLs and 5-mature miRNAs G content and their tendency to
147 act either as tumor suppressive miRs or oncomiRs, respectively.
148 In order to define the function of those tumor suppressive miRs, which have
149 relatively high G content in their TLs, we selected the 30 most TL-G-enriched
150 miRNAs, (over 35% G), retrieved their identified direct target genes from the
151 PubMed articles (Supplementary Table 2), and searched for biological processes
152 enrichment. Functional annotation was performed using the DAVID tool, and among
153 Gene Ontology (GO) biological processes; negative regulation of cell death,
154 regulation of signal transduction, and positive regulation of cell migration, were the
155 most significant ones in lung and breast cancers (Figure 3). The results show that a
156 large number of target genes of these tumor suppressive miRs are known proto-
157 oncogenes (MYC, MYCN, EGFR, ERBB2, ERBB3, ERBB4, AKT1, CCND1,
158 KRAS, SRC, BCL2, PIK3CA, MET, AXL, PDGFRB, JAK2, HMGA2, MYB,
159 AGR2, PRKCA, PIM1, RAF1, RHOA, MKL1, SALL4) (Supplementary Table 3). In
160 several cases, these oncogenes were common targets to multiple miRNAs, such as in
161 KRAS (miR-30c, -143, -193a, -200c, let-7a), CCND1 (miR-34b, -145a, -195, let-7e),
162 and EGFR (miR-34a, -143, -218) (Supplementary Table 3).

163 **Discussion**

164 Over the past two decades, it has become increasingly apparent that loss- or gain-of-
165 function of specific miRNAs contributes to cellular transformation and tumorigenesis
166 [23]. OncomiRs typically function to promote cell growth, inhibit apoptosis and

167 control the cell cycle, while tumor suppressive miRs function to inhibit cell growth
168 and induce apoptosis [24], and there are several examples in which a specific miRNA
169 can act either as a tumor suppressor or an oncomiR, depending on cellular context and
170 type of cancer [25]. However, a global miRNA downregulation in cancer was also
171 observed, and therefore, understanding the mechanisms driving miRNA
172 downregulation is important in uncovering the regulatory role of miRNAs in cancer
173 biology [26].

174 The results presented here support previous studies showing the potential
175 importance of TL-G content to the regulation of miRNAs expression and function
176 [19-22]. We have previously suggested that estrogens metabolites inside the lungs, as
177 a result of CS exposure, can potentially cause the observed widespread
178 downregulation of miRNA expression, and contribute to lung tumor development [27,
179 28]. Estrogen metabolism generates highly reactive metabolites, mostly catechol
180 estrogen-3,4-quinones, which form carcinogen depurinating G adducts [29].
181 Moreover, oxidative metabolites of estrogens can cause oxidative damage to G,
182 because of its lowest oxidation potential, by forming 8-oxo-dG (8-Oxo-2′-
183 deoxyguanosine), which eventually leads to carcinogenesis [30]. Repressed miRNAs
184 in lung and breast cancers were shown to be the most enriched in TLs G [22], and the
185 relation of estrogen to the development of these types of cancer is well documented
186 [27, 30, 31]. Furthermore, like in the current study, also in the aforementioned studies,
187 dual GG enrichment in miRNA TLs was even more pronounced [20-22]. Remarkably,
188 experimental studies have shown that sequences with repeated G bases (GG or GGG)
189 show higher reactivity toward oxidation than isolated G bases [32].

190 Despite analyzing during this study relatively small fraction (~14%) of the total
191 number of currently identified human miRNAs, our results indicate that the number of

192 tumor suppressive miRs is greater than oncomiRs. This is in agreement with the
193 finding that many of the known mutated genes are oncogenes [33], and with the
194 general consideration of miRNAs as safeguards of the genome [24]. Of note, a
195 remarkable redundancy was observed between the target oncogenes of the tumor
196 suppressive miRs (e.g. KRAS). Synergetic effects of functionally related tumor
197 suppressive miRs, that share common targets and control similar processes, were
198 shown before [34].

199 As indicated above, tumor suppressive miRs tended to be more G-enriched in
200 their TLs. G enrichment in tumor suppressive miRs TLs could affect binding of RBPs
201 such as Lin28, KSRP, and the heteronuclear ribonucleoprotein A1 (hnRNP A1),
202 which compete with KSRP for their common G-rich target sequence [35]. Notably,
203 hnRNPA2B1, another splicing factor, binds exosomal miRNAs through the
204 recognition of G-enriched motifs to control their loading into exosomes [36]; small
205 membranous vesicles which enable genetic exchange between cells, can transfer
206 functional miRNAs to recipient cells and consequently downregulate the expression
207 of their target genes [37]. It is noteworthy that sumoylation modification controls the
208 binding of both KSRP and hnRNPA2B1 to miRNAs [19, 36].

209 OncomiRs, on the contrary, have lower G content in their TLs, and are relatively
210 more G-enriched in their 5-mature miRNA forms. The differences in G enrichment
211 shown here between the 5 and 3-mature miRNAs might be attributed to the
212 differences between the functional guide strand and the passenger strand of mature
213 miRNAs, as one of the characteristics of human miRNA guide strands is excess of
214 purines [38]. This observation could also be related to the process of sorting miRNAs
215 into the exosomes, as it was shown that G-rich sequence is a dominant feature of
216 exosome-dominant miRNAs, suggesting the possibility that RBP-mediated

217 translocation of cellular miRNAs into exosome cargos occurs by G-recognition [39].
218 Indeed, oncogenic exosomal miRNAs (miR-17, -21, -106a, -155, -191) were highly
219 induced in lung cancer [40, 41], and chemoresistance of breast cancer cells was
220 recently shown to occur through the G-enriched miR-155 oncomiR exosomes
221 delivery [42].

222 **Conclusions**

223 Taken together, the current and previously published results suggest that G content of
224 miRNAs is an important feature determining their expression and function. MiRNA
225 TL-G enrichment may have important role in the carcinogenic process by affecting
226 the observed global downregulation of tumor suppressive miRs, which cause
227 induction of their target oncogenes, and commit cells towards carcinogenesis. On the
228 other hand, high G content in the oncomiRs 5-mature sequences may function in
229 guide-strand selection and miRNA targeting into the exosomes. Elucidating the
230 molecular mechanisms that involve miRNAs G can have major implications for
231 cancer research, therapy and prevention [43, 44].

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237 **Competing interest**

238 The authors declare no conflict of interest.

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360 **Figure legends**

361 **Figure 1.** Number of PubMed articles describing the function of tumor
362 suppressive miRs and oncomiRs relative to (a). TL G enrichment. (b). 5-mature G
363 enrichment.

364 **Figure 2.** G enrichment of tumor suppressive miRs relative to oncomiRs. Single,
365 dual and triple G were calculated in SLs, TLs, 5 and 3-mature miRNAs involved
366 in lung and breast cancers.

367 **Figure 3.** Enriched GO terms of the target genes of G-enriched tumor suppressive
368 miRs. Shown are significant –log2 Benjamini p-values of the biological process
369 terms (level 5) of the 30 most TL-G-enriched tumor suppressive miRs target
370 genes, as were identified using the DAVID functional annotation tool.

371 **Supplementary Material**

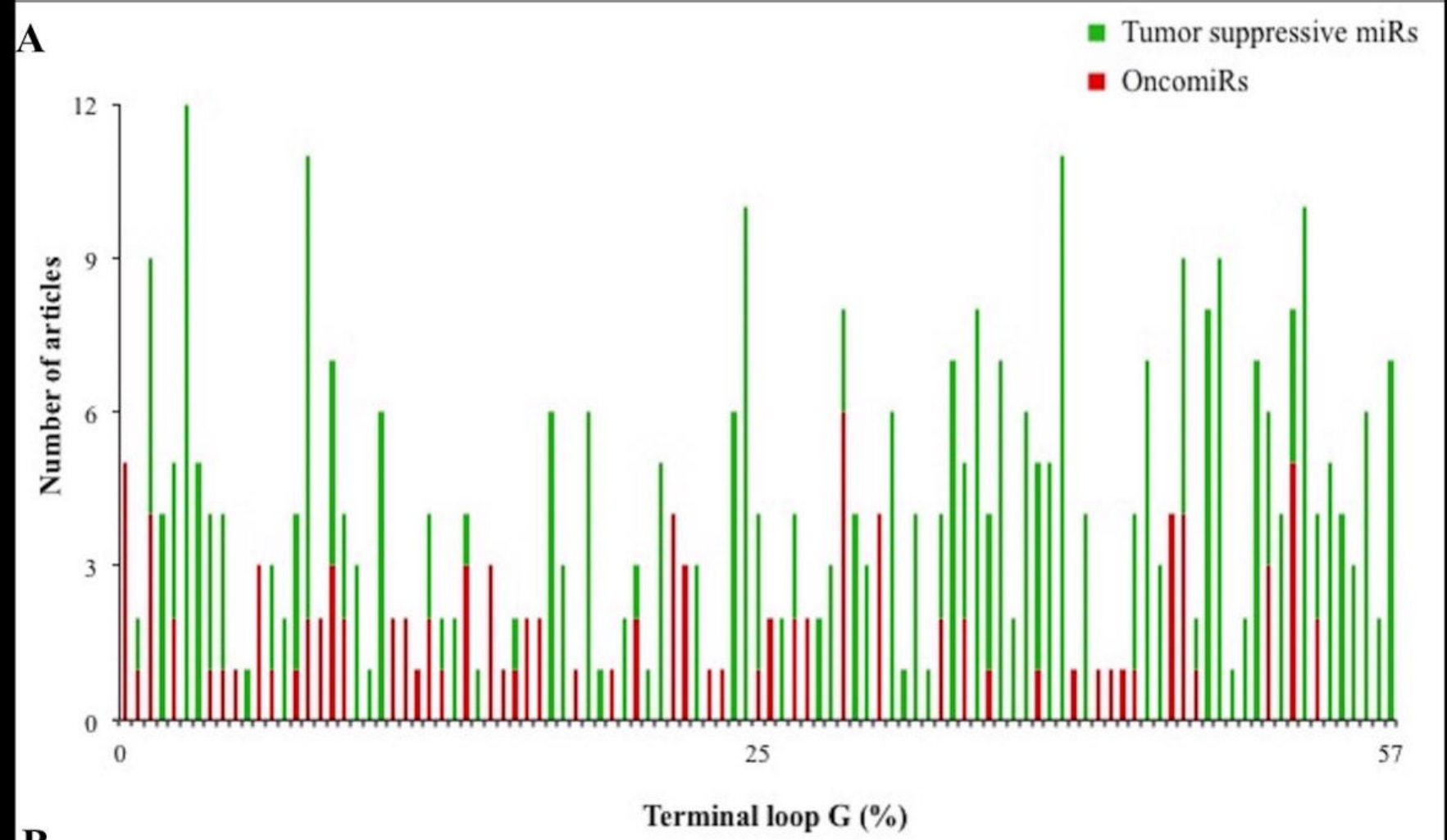
372 **Supplementary Table 1:** SLs, TLs, 5 and 3-mature miRNA sequences, and their
373 G enrichment. Shown are 255 miRNA sequences retrieved from the miRBase
374 database. G enrichment of sequences represents the ratio of G number relative to
375 total nucleotide number.

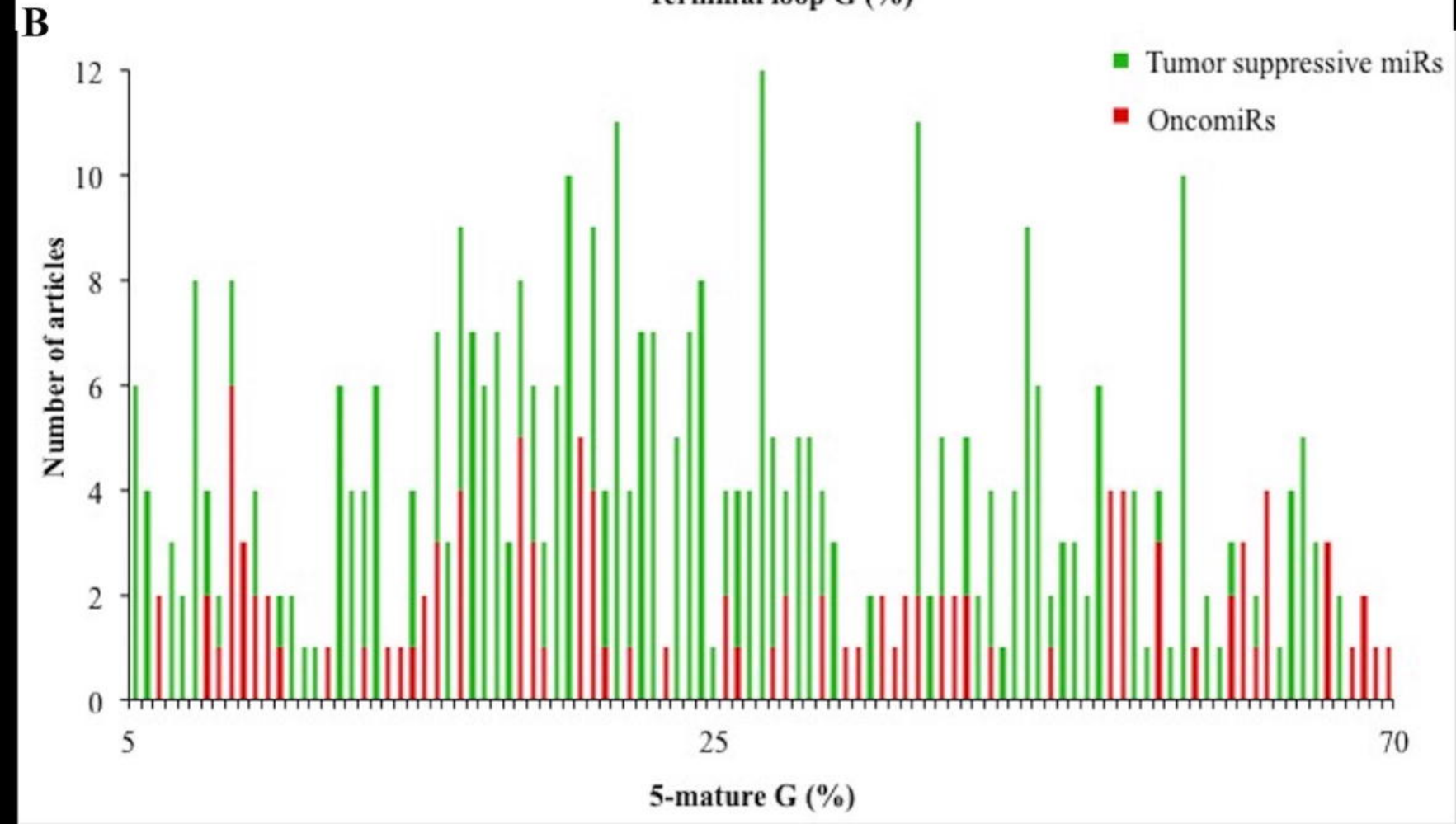
376 **Supplementary Table 2:** Tumor suppressive miRs, oncomiRs, and the references
377 from PubMed Database describing their function in lung and breast cancers.
378 When search in PubMed had no results it appears as unknown function.

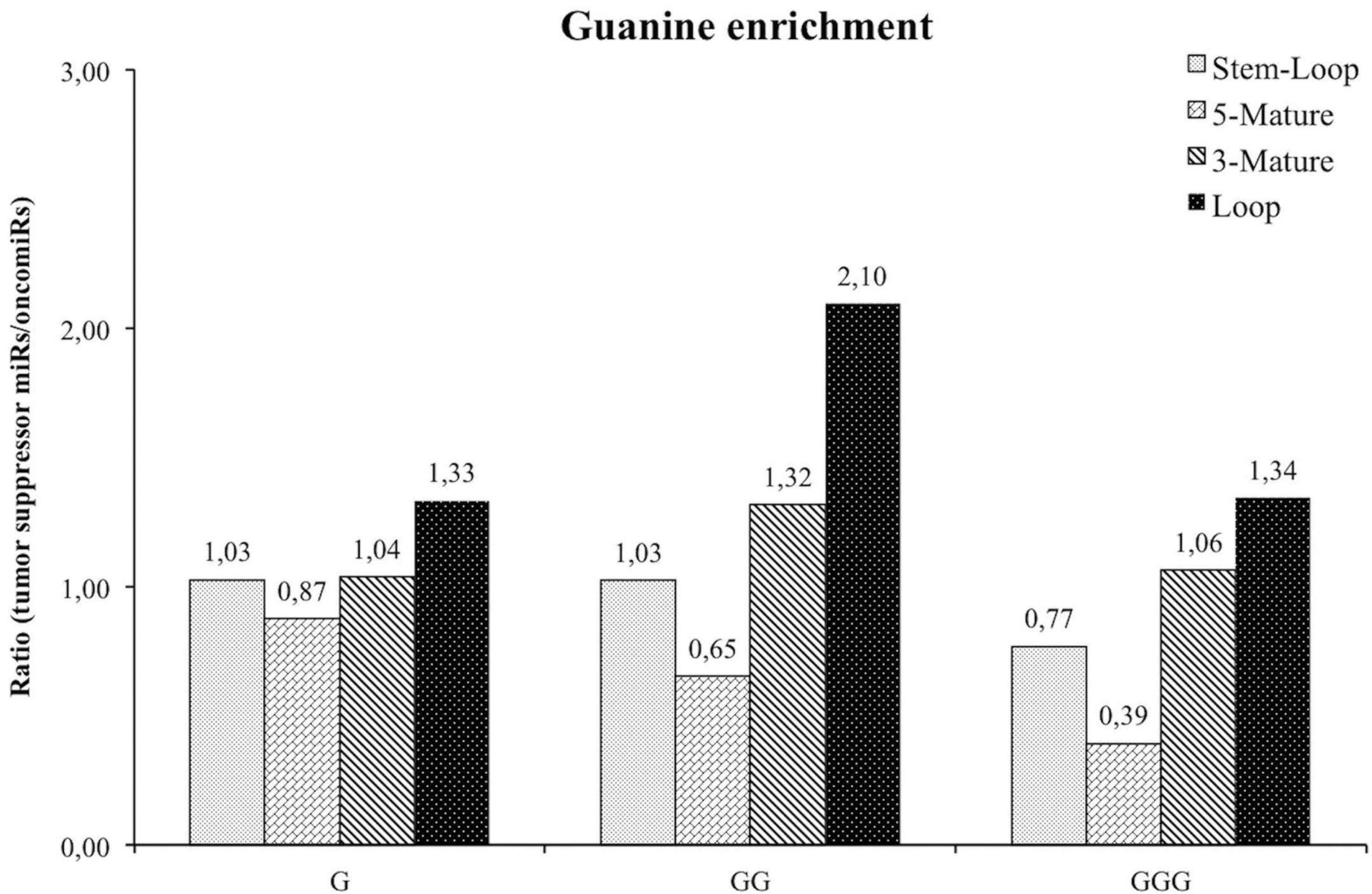
379 **Supplementary Table 3:** G-enriched tumor suppressive miRs target genes.
380 Shown are 30 most TL-G-enriched tumor suppressive miRs and their direct target

381 genes in lung and breast cancers, as were described in the references from

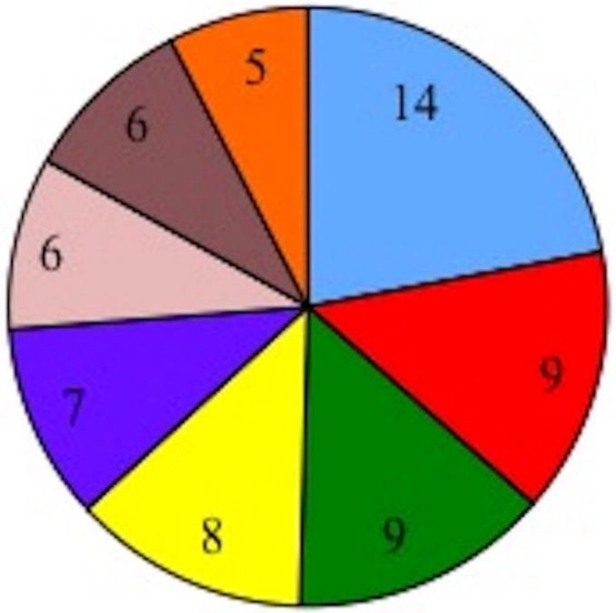
382 PubMed Database (Supplementary Table 2).


A
Tumor suppressive miRs
OncomiRs
Number of articles
12
9
6
3
0
0
25
57
Terminal loop G (%)

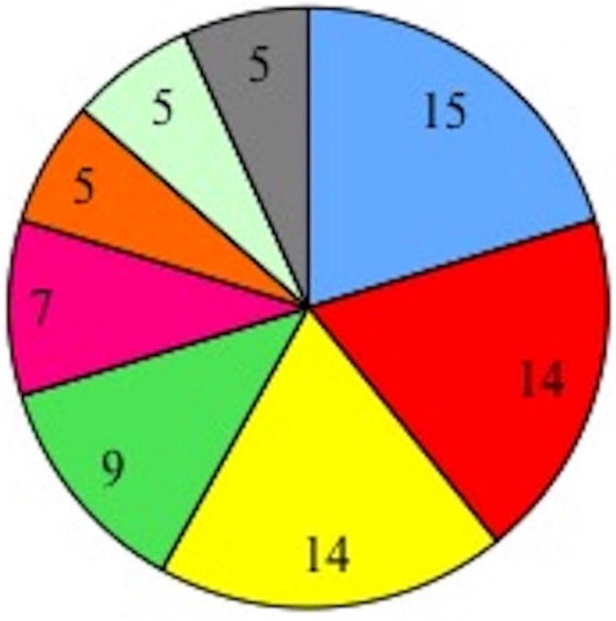

B
Tumor suppressive miRs
OncomiRs
Number of articles
12
10
8
6
4
2
0
5
25
70
5-mature G (%)


Guanine enrichment
Stem-Loop
5-Mature
3-Mature
Loop
Ratio (tumor suppressor miRs/oncomiRs)
3,00
2,00
1,00
0,00
1,03
0,87
1,04
1,33
1,03
0,65
1,32
2,10
0,77
0,39
1,06
1,34
G
GG
GGG

Breast cancer



Lung cancer



- Negative regulation of cell death
- Regulation of signal transduction
- Immune system development
- Positive regulation of cell migration
- Reproductive structure development
- Regulation of angiogenesis
- Mesenchyme development
- Response to steroid hormone
- Positive regulation of smooth muscle proliferation
- Respiratory system development
- Regulation of mitotic cell cycle
- Regulation of DNA repair