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Indirect role of microRNAs and transcription factors in the regulation of important cancer genes: A network biology approach

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Abstract

Cancer is one of the leading causes of death worldwide. Although the mechanisms of gene regulation in cancer have been the subject of intense investigation during the last decades, the precise role of regulatory processes in cancer is largely unknown. More specifically, it is not completely understood how microRNAs and transcription factors regulate and influence the cancer-related processes. In the present study, using cancer-specific biological networks we examine the role of microRNAs and transcription factors (TFs) in regulation of important cancer genes. The importance measures which are used in this study consider both network structure information and biological data on miRNA- and TF-based gene regulation. By analyzing cancer-specific PPI, signaling and metabolic networks, it was shown that microRNAs and transcription factors tend to regulate those genes which are in the neighborhood of important components of cancer-specific PPI, signaling, and metabolic networks. The role of microRNAs was found to be particularly important, which confirms our previously-published results on the importance of microRNAs in detecting important network components. Moreover, we highlight that the miRNAs appear to apply their function via regulating the "neighbors" of important cancer genes, which implies their indirect role in cancer, and presumably, in fine-tuning the effect of other cancer-related genes.

Key words: Network biology, Regulation by miRNA, Regulation by transcription factors, Carcinogenesis.

Introduction

The expressions of genes are regulated at different levels by respective factors. At transcription level the regulation exerted by DNA elements and factors acting upon them leads to differential gene expression. The genomic elements and factors involved are very diverse (1-3). Regulation of promoter elements by diverse combination of different transcription factors (TFs) constitutes the basis of transcription which is tissue- and development-specific. The expression of genes is also controlled by the chromatin state encompassing the genes through epigenetic mechanisms (4). Many of these also are correlated with different signal transduction pathways, which makes the scene of action very complicated. On the other hand, in most cases translation of an mRNA to the respective phenotype is dependent on other regulatory pathways. Post-transcriptionally, mRNAs are controlled by a plethora of noncoding RNAs (5, 6). Among these new players are microRNAs (miRNAs) emerging as important regulatory molecules which control the level of translation of respective target mRNAs (7, 8).

MicroRNAs are small, ~22 nt long noncoding RNAs comprising 1-2% of genes in eukaryotes (9, 10). They are located both intra- and intergenically throughout the genome. A considerable proportion of miRNA genes are organized in clusters and are transcribed as polycistronic products (11), which is presumably related to their interrelated functions in targeting the same mRNA(s) in a given cell or particular developmental stage. With some exceptions, miRNAs are transcribed by pol II and undergo those processing steps observed in case of mRNAs (8, 9). Thousands of miRNAs are discovered in human and also in eukaryotic model organisms. The fact that each miRNA can interact with hundreds of mRNAs demonstrates their vital role in normal gene regulation and disease pathogenesis where dysregulated.

MiRNAs are transcribed as a long primary miRNA which then processed by nuclear RNase III Drosha to a hairpin-shaped 70-120 nt pre-miRNAs. Consequently, pre-miRNA, exported to cytoplasm, is further processed by RNA induced silencing complex (RISC) to a 22 nt mature miRNA (12). RISC guides the single stranded miRNA to its mRNA targets at their 3'UTR for translational attenuation of targeted transcripts. The target recognition is not done through perfect match between miRNA-mRNA, but dictated only by a 7 nt-mer seed site (nucleotides 2-8 from 5' end of miRNAs) as evidence implies, though the context of the seed site may be of importance as well. Due to its imperfect match to the target sequences many miRNAs can target multiple targets and any target in turn may have multiple recognition sites for the same or different miRNAs (13, 14). These make miRNAs perfect players in gene regulatory networks as by differential interaction of many miRNAs with their respective and potential targets help the system to elaborate desired fates and sustain in both normal and distressed situations. According to one study only one third of miRNAs studied had tissue or cell specific expression (13).Therefore, the majority of miRNAs are expressed nonspecifically and it appears that the relative

expression of ubiquitous miRNAs varies among tissues indicating other factors may contribute in differential relative expression of ubiquitous miRNAs.

Biological robustness refers to the ability of biological systems to remain stable and functional despite various internal and external perturbations. Robustness is an intrinsic property of biological systems at any level of their organization, from the regulation of a single gene expression in a cell to the survival of the organism as a whole (15, 16). Robustness explains both the stability of living organisms and their evolvability in the context of internal and external fluctuations. Variability and complexity is the major sources for remaining robust. To remain stable (and at the same time changeable), organisms require a complex network of robust contents, and robustness has to be seen in the context of numerous dynamic pathways and networks (17, 18).

Breaking down the biological systems into their components may not be useful in studying robustness which is an emergent property of the system as a whole, because there are many redundant components which can compensate for each other. In many cases, this phenomenon is attributed to the functional degeneracy of major components of living systems at molecular level (18, 19). Degeneracy is common in all levels of biological organization, from the genetic code itself to transcriptional and post-transcriptional regulatory machines (20, 21). Degeneracy (and robustness) is well discernible at transcriptional regulation by TFs and the epigenetic (soft inheritance) regulation, including translational control of mRNA targets by numerous diverse miRNAs (18, 22). TFs and miRNAs and their regulatory effects are the focus of this study.

Expression of every gene is primarily controlled by TFs at the transcriptional level and ultimately is fine-tuned by miRNAs regulating mRNA translational rate. Both miRNAs and TFs work cooperatively (and competitively when requires) to determine the differentiated state of cells in normal state and in response to intrinsic and extrinsic disturbances (23). While TFs work generally as switch-on and -off regulators, miRNAs modulate translation of respective targets via many weak interactions which is an intrinsic property providing robustness to biological systems (24). This is in line with the findings that many miRNAs, when knocked down, show no apparent effect. This observation indicates the weak (but maybe additive) effects of miRNAs regulating biological processes (24).

Cancer is currently the second leading cause of death worldwide (25). Currently, over 3 million cancer related publications are available in PubMed, which account for 12% of the whole biomedical literature indexed in this database. Though research on cancer witnessed an exponential growth during the last decades, the mechanisms underlying cancer development seem to be remained unexplored to a great extent. As mentioned above, the disparity can be attributed, at least in part, to the fact that biological research is mainly devoted to the study of biological components, rather than biological systems. Consequently, comprehensive systems-level approaches to study biological systems, e.g. cancer cells, may be able to explain the complex phenotypes which are otherwise impossible to understand (26).

In a previous study (27), we defined a miRNA-based measures for gene importance, TAmiC, and then using this measure we investigated how miRNAs tend to regulate important vertices (or equivalently, genes) of different biochemical networks. In the present study, we show that TAmiC can successfully predict important genes in human cancer networks. The success of this measure is independent of the nature of the network. Additionally, TAmiC performs significantly better than other similar measures (based on the number of TFs that regulate a gene). Our results highlight the role of miRNAs in regulating the genes involved in cancer.

Materials and methods

Cancer networks

In the present study, a number of human cancer-specific networks are investigated:

- Cancer signaling network: A manually curated human cancer signaling network, including 1,634 nodes and 4,665 signaling regulatory relations, has been previously reported (28). In the present work, we applied the undirected graph underlying this network.
- Cancer metabolic network: The generic metabolic network of cancer cells (29) was used in the present study. A constraint-based model of a metabolic network is essentially based on a "hypergraph" model (and not a "graph" model) of metabolism (30). Therefore, we decided to convert this network to a "reaction-centric" metabolic network, which is a graph representation of the network (31). Application of a reaction-centric metabolic model enables us to directly connect the regulatory factors (i.e., TFs and miRNAs) to the network nodes, which are metabolic enzymes in this case. The reaction-centric network representation of cancer metabolic network includes 2,302 vertices (reactions) and 90,674 edges (linking metabolites).
- Cancer protein-protein interaction (PPI) network: In the present work, we used the PPI network of muscle bladder cancer (32). This network has been reconstructed based on literature-mining and includes 286 vertices (proteins) and 661 edges (interactions).

MiRNA Targets

The genome-wide predicted human miRNA target genes were obtained from the TargetScanS web server (version 6.2) (33). This dataset contains a total of 11,161 genes regulated by 1,537 miRNAs (grouped in 153 conserved miRNA families). As an independent dataset of miRNA targets, we also used the dataset of predicted miRNA target genes obtained from PicTar (34). The latter dataset includes 6,243 genes regulated by 168 conserved miRNAs.

It should be noted that it is also possible to use the high-confidence miRNA targets obtained by HITS-CLIP experiments (35). Although application of such data in our analysis resulted in patterns comparable to the application of predicted miRNA targets (data not shown), we decided not to use the HITS-CLIP data in our analysis. The reason is that, these experiments tend to neglect low copy number miRNAs, and therefore, the results are biased toward the behavior of high copy number miRNAs.

TF Targets

In the present work, we used two datasets of predicted human TF targets:

- *The Corà dataset* (36), including 9,348 target genes. This dataset is obtained by an algorithm which combines human and mouse genomic data, sequence over-representation data and gene co-regulation data.
- *The Xie dataset* (37) including 14,861 target genes. This dataset is obtained by comparative analysis of human, mouse, rat and dog genomes.
- In each of the two datasets, the number of TFs that can (potentially) regulate each gene is determined.

Mutation rates of genes

A dataset of human genes along with their nonsynonymous to synonymous substitution rates (d*N*/d*S*) are obtained from Colombo *et al.* (38). Briefly, these values have been obtained by comparing 35 sets of orthologous genes across four genomes, namely human, chimpanzee, gorilla, and orangutan.

Essential genes of human

A gene can be considered as "essential" in human if it is associated with a life-threatening disease phenotype, which typically results in death before puberty (39). A list of human essential genes were obtained from DEG database (40, 41) (available from http://tubic.tju.edu.cn/deg/). DEG v10.6 includes 2570 human essential genes.

Human oncogenes

A list of 780 candidate oncogenes are previously reported by Khosravi and coworkers (42). This reference list of oncogenes was used for validating the list of top cancer genes discovered computationally.

Measures of importance

In this study, several measures of importance are compared. A detailed list of these measures and their mathematical descriptions are presented in Ref. (27). In the present work, eigenvector centrality was computed using *igraph* software package (43) (available from http://igraph.org), while degree, betweenness and closeness centrality measures are computed using *NetworkX* (44) (available from https://networkx.github.io).

In our previous work (27), three miRNA-based measures of importance, namely total miRNA count (miRcount), total adjacent miRNA count (TAmiC) and average adjacent miRNA count (AAmiC) are defined. Then, using these measures, we investigated how miRNAs tend to regulate important vertices of different biochemical networks. In the present work, the possibility of miRNA role in the regulation of important nodes in cancer-specific networks is investigated. Additionally, one may ask whether TFs have a comparable role in regulation of important nodes in cancer networks. Therefore, analogous to the miRNA-based measures (27), we define three TF-based measures of importance. Suppose that t_i is the number of TFs that regulate (the gene in) node i of the network, and $\Gamma(i)$ is the set of nodes adjacent to node i, and $T^*(i)$ be the subset of neighbors of i that are regulated by at least one TF. Then,

- similar to "miRcount", we define "TFcount" as the number of TFs that regulate node i;
- similar to "TAmiC", total adjacent TF count (or simply "TAtfC")is defined as $\sum_{j\in\Gamma(i)} t_j$;
- similar to "AAmiC", average adjacent miRNA count (or simply "AATFC"), is defined as $\sum_{j\in T^*(i)} t_j / |T^*(i)|$.

Attack robustness

In the present work, we studied the attack robustness of biological networks as described previously (27, 45, 46). In this type of study, network vertices are removed consecutively (either in a random order, or alternatively, in the order of their "importance" in the network). Then, the size of the largest connected component of the network is considered as a measure of network robustness. The more important the removed network vertex, the smaller the size of the largest connected component of the network. Different measures of importance are suggested in the literature (see above). Consequently, the choice of the importance measure can influence the size of the largest connected component. Removing the network vertices at random order might help to understand the effectiveness of each importance measure.

Results and Discussion

In a previous work (27), we showed that the TAmiC measure can successfully predict biologically important nodes of a network. Here, we show that the application of this measure can be extended to the analysis of cancer cells.

Finding important genes in cancer networks using TAmiC, TAtfC and degree

First of all, we checked the ability of different importance measure in detecting important nodes in cancer networks. The idea is that, if a measure can successfully detect importance vertices of a network, by removing these vertices (based on the order of their importance) it is possible to "damage" a network. On the contrary, if the measure does not successfully predict the important vertices, their removing would not damage the network beyond what is expected by chance.

Figure 1 shows the effect of removing vertices from the cancer networks when the vertices are removed randomly, or removed based on the order of the six miRNA- and TF-based importance measures. From this figure, it is obvious that TAtfC and TAmiC are the best measures in detecting important vertices in PPI, signaling and metabolic cancer networks. On the other hand, deleting nodes based on miRcount and TFcount is in fact comparable to deleting nodes at random. This observation presumably suggests that miRNA and TF do not directly regulate the important cancer genes, but they prefer to apply their regulation indirectly, i.e., by regulating the neighboring vertices of the important nodes.

To further investigate the properties of TAmiC and TAtfC, we compared these measures to the classical "centrality measures", which are based on network structure only. In Figure 2, performance of these two measures in detecting important nodes is compared with the performance of degree centrality, and in Supplementary Figure S1 this comparison is done for several other centrality measures. Interestingly, the behavior of TAtfC and degree (and to some extent that of TAmiC and degree) are found to be similar. One may ask whether these two measures contain additional information

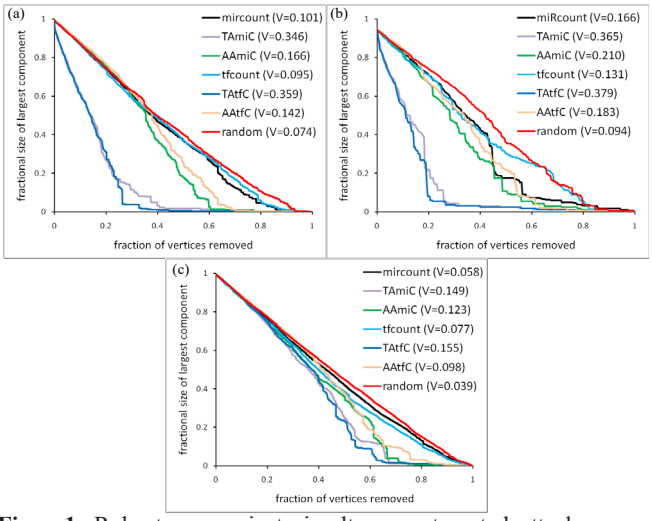

Figure1. Robustness against simultaneous targeted attack according to three TF-based importance measures (TFcount, AAtfC and TAtfC) and three previously defined miRNA-based importance measures (27), namely miRcount, AAmiC and TAmiC for: (a) cancer signaling network; (b) cancer PPI network; and (c) cancer metabolic network. The vulnerability (27) value, *V*, is also shown in each case. The miRNA and TF targets were obtained from PicTar and Corà datasets, respectively. The "random" results were obtained by 10 times repeating a random attack (i.e., by deleting network vertices at random) and taking the average.

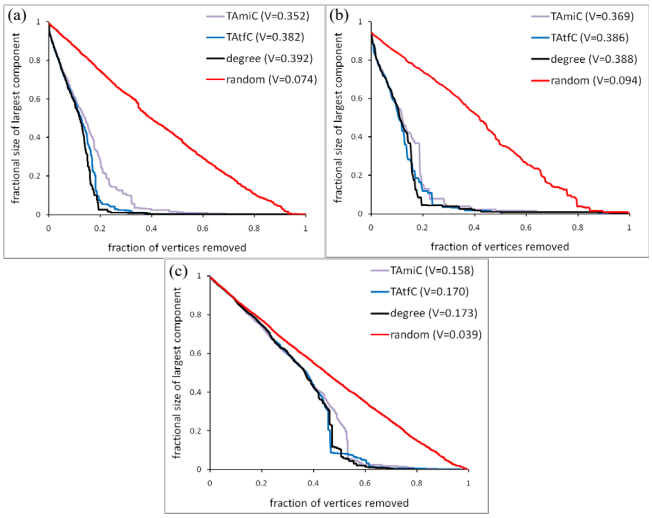

Figure 2. Robustness against simultaneous targeted attack according to TAmiC, TAtfC and degree centrality measure for: (a) cancer-signaling network; (b) cancer-PPIN and (c) cancer-metabolic network. The miRNA and TF targets were obtained from TargetScanS and Xie datasets respectively. The "random" results were obtained by 10 times repeating a random attack (i.e., by deleting network vertices irrespective of their importance) and taking the average. It should be noted that the more complete view of this analysis including robustness graphs based on the other centrality measures comprising betweenness, closeness and eigenvector centralities besides those reflected in this figure has been shown in Figure S1 of the supplementary files.

compared to degree.

To better show the relationship between TAmiC, TAtfC and the classical centrality measures, pairwise correlation between these measures are presented in Table 1. Note that TAtfC and degree are highly correlated, which suggests that TAtfC and degree are interchangeable.

Important genes in cancer networks based on their mutation rates

In evolutionary biology, d*N*/d*S* represents the ratio of the number of nonsynonymous substitutions to the number of synonymous substitutions. This value is a measure of selective pressure acting on a protein-coding gene. In other words, an important protein with "conserved" sequence must have a greater d*N*/d*S* value.

In the present work, we investigated the possible correlation between the importance of proteins in cancer and their "conservedness" based on d*N*/d*S*. For this purpose, we used a previously reported dataset of human proteins (38). We compared the conservedness of these proteins to the importance rank of proteins in the human cancer metabolic network. Figure 3 summarizes the results. Although in case of all of the three measures a negative correlation is detected, one can observe that only in case of TAmiC a significant correlation is observed between importance rank and d*N*/d*S* ($p<0.01$). In case of TAtfC and Degree, the correlation was not statistically significant ($p>0.05$). In conclusion, TAmiC is statistically correlated with the conservedness of proteins in metabolic cancer network, which again confirms the success of this measure in correctly predicting the biologically-relevant important genes.

As mentioned above, TAtfC and degree are highly correlated. Therefore, in cases like Figure 3 where degree fails to outperform other importance measures, TAtfC also fails in accord, as it seems behaving almost similarly.

The top important cancer genes detected by TAmiC

In the next step, we analyzed the top genes which are removed from the three networks based on TAmiC, TAtfC and degree. It is expected, in general, that top-ranking important genes of a biological network are more "essential" compared to other genes. Figure 4 shows how frequent are the essential genes among the top-ranking network vertices. Interestingly, the results show that the success rates of TAmiC, TAtfC and degree are almost similar. As an example, the top 50 genes of signaling network are mentioned in Supplementary Table S1, which shows that many of the genes are shared by the three top-ranking gene lists. Note that these results are fundamentally different from those we reported in our previous work (see Fig. 3A of Ref. 27), where TAmiC was found to be significantly more suc-

Table1. Correlations between TAmiC, TAtfC and the four centrality measures for cancer signaling network, cancer metabolic network. TAmiC and TAtfC are computed based on TargetScanS and Xie datasets.

	Degree		Betweenness		Closeness		eigenvector		TAmiC & TAtfC
	TAmiC	TAtfC	TAmiC	TAtfC	TAmiC	TAtfC	TAmiC	TAtfC	
Cancer signaling network	0.821	0.910	0.702	0.809	0.602	0.568	0.603	0.560	0.814
Cancer metabolic network	0.949	0.978	0.730	0.729	0.893	0.901	0.901	0.910	0.966

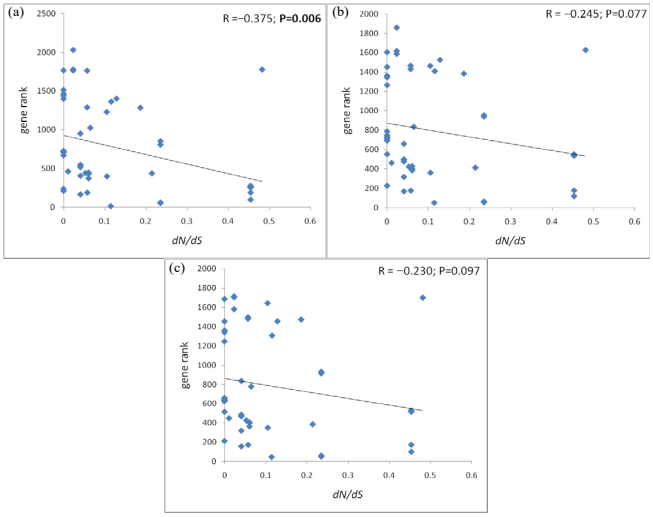

Figure 3. Correlation between *dN/dS* and the importance rank of genes based on TAmiC (a), TAtfC (b) and degree (c) in cancer metabolic network. The Pearson correlation coefficient and their associated *p*-value are also shown on the plots.

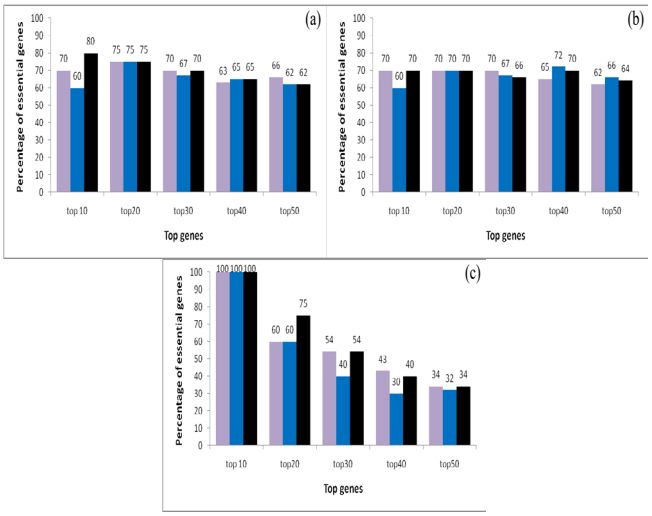

Figure 4. Percentage of essential genes among important ones identified by TAmiC (violet), TAtfC (blue) and degree (black) through vulnerability analysis of (a) cancer signaling network; (b) cancer PPI network; and (c) cancer metabolic network.

cessful in detecting the essential genes. The difference is presumably due to the difference in the definition of "essential genes" in yeast and human. In yeast, knockout analysis has been done practically for every single gene in the genome and those mutants with "no-growth" phenotype are considered as essential (47). On the other hand, in haman, genes with clinical features of death before puberty are considered to be essential (39).

When we focused on the list of the top genes detected by TAmiC, we observed that several important genes in cancer are among the top genes. To test whether this overrepresentation is significant, we performed a series of statistical analyses. As an example, when we analyzed the list of TAmiC-based top 50 genes of the cancer signaling network, we observed that 30 genes (i.e., 60%) are in the reference oncogene list (reported in Ref. 42). Then, we generated 20 gene lists, each including 50 randomly selected genes. In all of the cases, the number of oncogenes were less than 30. The results suggest a significant overrepresentation of cancer-related genes in the list of TAmiC-based top genes (p<0.001, one-sample Wilcoxon signed rank test). Similar results are observed in case of other networks, which shows the significance of our findings.

Based on the analysis of the cancer metabolic network, the highest ranking gene was fatty acid synthase (Entrez ID: 2194), which has been introduced as a potential therapeutic target in cancer (48). The second best ranking gene is ELOVL fatty acid elongase 4 (Entrez ID: 6785), whose methylation level is recently found to be linked to hepatocellular carcinoma (49). The third best ranking gene, transmembrane protein 54 (Entrez ID: 113452), has been also reported to be associated with different cancer types (50, 51).Up-regulation of the fourth gene in the list, AMPD3 (Entrez ID: 272) has been reported to be involved at least in some lung adenocarcinomas (52). The fifth gene in this list, serine palmitoyltransferase (SPTLC2, Entrez ID: 9517), is reported to be linked to breast cancer (53).

Based on the analysis of signaling network, the highest ranking gene was androgen receptor, whose role in cancer is extensively studied (54, 55). Furthermore, the other top ranked genes in this list, including SMAD3 (56), SRC (proto-oncogene non-receptor tyrosine kinase) (57), AKT1 (v-akt murine thymoma viral oncogene homolog 1) (58) and SMAD4 (59) also have well-known roles in carcinogenesis mechanisms.

Based on the PPI network, the top ranked genes are: JUN (jun proto-oncogene) (60), tumor protein p53 (61), EGFR (epidermal growth factor receptor) (62), RB1 (retinoblastoma 1) (63) and E2F1 (E2F transcription factor 1) (64). All these genes have putative roles in cancer.

By comparing the list of top genes obtained by TAmiC, TAtfC and degree, it was observed that in case of signaling and PPI networks, best ranked genes at the top of the three lists are similar. However, in the case of cancer metabolic network, top genes showed variations. We conclude that the indispensable role of TAmiC for detecting important cancer genes can potentially be applied for the discovery of new promising drug targets for cancer.

Other articles in this theme issue include references (65-76).

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