Abstract. During malignant progression to overt cancer cells, normal cells accumulate multiple genetic and non-genetic changes, which result in the acquisition of various oncogenic properties, such as uncontrolled proliferation, drug resistance, invasiveness, anoikis-resistance, the ability to bypass oncogene-induced senescence and cancer stemness. To identify potential novel drug targets contributing to these malignant phenotypes, researchers have performed large-scale genomic screening using various in vitro and in vivo screening models and identified numerous promising cancer drug target genes. However, there are issues with these identified genes, such as low reproducibility between different datasets. In the present study, the recent advances in the functional screening for identification of cancer drug target genes are summarized, and current issues and future perspectives are discussed.

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2. Types of functional screening according to phenotypes used
3. Conclusions and future perspectives

1. Introduction

Most human solid tumors develop through multi-step carcinogenesis (1,2). During this process, normal cells, presumed to be tissue-specific stem cells, accumulate multiple molecular changes advantageous to step-wise growth, finally transforming into overt cancer cells (3). Previous advances in genome-wide profiling technologies have revealed a number of molecular changes in malignant cells at the genetic, epigenetic, transcriptional and translational levels (4-6). Genetic alterations in proto-oncogenes that significantly contribute to malignant phenotypes are called driver oncogenes, such as activated forms of epidermal growth factor receptor (EGFR), HER2/Neu and BRAF (7-10). There are multiple drugs that successfully target these driver oncogenes. For example, EGFR-targeted drugs, such as gefitinib and erlotinib, exhibit anti-cancer activity against lung cancer with activating mutations in EGFR (11,12). However, it is difficult to pharmacologically inhibit oncogenic signaling of some driver oncogenes. For example, the development of mutant KRAS-targeted drugs has proven problematic over the previous three decades (13). Although recently, treatment with AMG510, a novel inhibitor against KRAS G12C, resulted in a promising response rate in patients with lung cancer harboring this specific type of mutation, development of drugs targeting other types of KRAS mutations have not yet been successful (14-16). In addition, mutations in driver oncogenes in a number of types of human cancer have not been identified (17). In such cases, cancer results from non-oncogenes conferring various malignant phenotypes, occasionally in a context-dependent manner (18) and these genes may serve as novel therapeutic targets. For example, a study demonstrated that cancer cells depend on non-oncogene Heat shock factor 1 (HSF1), which is the master regulator of the heat shock response in eukaryotes, for their proliferation and survival than their non-transformed counterparts (19). To identify drug target genes for cancer cells harboring oncogenes which are difficult to pharmacologically inhibit, or do not have known oncogenes, it is vital to perform an unbiased, large-scale functional screening (20). Two important gene modulating technologies, RNA interference (RNAi) and clustered regularly interspaced short palindromic repeats-associated protein 9 (CRISPR-Cas9) have emerged as powerful tools for evaluating gene function (21). In addition, technologies in next generation sequencing have improved. The combination of these advanced technologies has allowed investigation of gene function at genome-wide levels in a high-throughput manner.

Thus, functional screening based on cancer-specific characteristics has been extensively conducted. In the
In cancer cells (24). Using this approach, two housekeeping genes have been identified, proteasome 20S subunit alpha 6 (PSMA6; a proteasomal catalytic subunit) and eukaryotic translation initiation factor 2 subunit beta (eIF2β); a subunit of translation-initiation factor eIF2), as promising therapeutic targets for lung cancer (30,31).

Another way to identify essential genes that contribute to oncogenic phenotypes is to reveal the genes which cancer cells depend on in specific contexts; for example, with certain types of driver oncogenes (32). This situation is referred to as synthetic lethality and is described later. One study demonstrated that an essential gene BUD31, a component of the spliceosome is a potential therapeutic target specifically in MYC-driven cancers (33).

### Synthetic lethality

A synthetic lethality refers to a phenomenon in which inhibition of one of two genes has no significant effects on cell viability but perturbation of both genes results in cell death (32). Synthetic lethality has attracted interest for the following reasons: i) If the synthetic lethality specifically occurs in cancer cells, treatments targeting genes involved in the synthetic lethality have a high therapeutic index; and ii) if the synthetic lethality involves driver oncogenes highly refractory to currently available treatment strategies, synthetic lethal genes may serve as good targets in types of cancer influenced by these oncogenes. A good example of such a gene is oncogenic KRAS, the most frequently mutated oncogene, although KRAS-targeted therapy is not used clinically (14). Using RNAi library screening, several studies have identified synthetic lethal genes in KRAS-mutated cancers, such as STK33, Tbk1, PLK1, SNAIL2, CDK1 and GATA2 (34-39). However, these identified genes rarely overlapped between studies (40) and the identification of a synthetic lethal effect caused by STK33 has not been reproduced (41,42). A recently conducted large-scale synthetic lethal RNAi screen, Project DRIVE, also failed to confirm significant synthetic interactions of mutant KRAS with these identified synthetic lethal genes (20). There are several possible reasons for such inconsistent results, including differences in methods of gene silencing (for example RNAi methodologies such as transient transfection of siRNAs or shRNA, and difference in types of library), and differences in types of cells used (for example variable dependencies on KRAS signaling). In particular, the latter seems to significantly influence screening results. Most studies of KRAS synthetic screens used cancer cell lines with or without mutant KRAS and/or isogenic cancer cell lines transfected with or without mutant KRAS (34-39).

Cancer cell lines are highly variable in genetic changes (even those with the same driver oncogenes), which may result in inconsistent screening results (17,43).

Project DRIVE comprehensively assessed dependencies and synthetic lethal relationships using 398 cancer cell lines from different organs (20). To minimize false-positive rates, an average of 20 shRNAs per gene were used and, although synthetic lethal genes could not be confirmed for mutant KRAS, a number of novel findings regarding synthetic lethality which are translatable to developing novel therapeutics were identified. For example, reduced expression levels of an anti-apoptotic protein BCL2L1, and increased expression levels of pro-apoptotic protein BIM, were the
strongest predictors of the growth-inhibiting effects following knockdown of anti-apoptotic protein myeloid cell leukemia sequence 1 (MCL1).

Recently, via genome-wide CRISPR-Cas9 screening, two independent groups identified WRN helicase as a synthetic lethal target in microsatellite unstable cancer types (44,45). Moreover, a small molecule inhibitor of WRN helicase (NSC617145) has been revealed to exhibit cytotoxic effects in cells derived from patients with Fanconi anemia, in a synthetic lethal manner (46).

**Dropout viability screening under drug treatment.** Drug resistance is a critical problem in chemotherapy (47). Cancer can be resistant to a number of types of drugs, such as cytotoxic, molecular-targeted drugs and immune checkpoint inhibitors (47-49). Therefore, researchers focus extensively on finding therapeutic approaches to overcoming the development of drug resistance.

Dropout viability screening in the presence of anti-cancer drugs is a powerful approach to identifying genes responsible for drug resistance and several potentially chemosensitizing targets have been reported (Table I). Using a genome-wide an arrayed RNAi library, Whitehurst et al (50) identified several genes influencing resistance to paclitaxel in a lung cancer cell line. Lin et al (51) identified MCL1 as a potential drug target gene that sensitizes a small cell lung cancer cell line to ABT-737, an inhibitor of the antiapoptotic molecules Bcl-2, Bcl-X(L) and Bcl-w. After the development of pooled RNAi library technology, numerous investigators began using such libraries. For example, Prahallad et al (52) revealed genes responsible for resistance to a BRAF inhibitor PLX4032 (vemurafenib) in types of cancer harboring BRAF V600E mutations. It was revealed that EGFR activation, which is rapidly induced by vemurafenib treatment, induces resistance to vemurafenib treatment, suggesting that combination therapy of vemurafenib and an EGFR inhibitor may be beneficial. Previously, studies using CRISPR‑Cas9 libraries were published. Most of these studies used the same type of genome-wide library, GeCKO CRISPR Library version 1 or 2, comprising of >120,000 sgRNAs targeting nearly the entire genome (53-56). For example, Sustic et al (56) identified the endoplasmic reticulum to nucleus signaling 1 (ERN1)-JNK-JUN pathway as a potential target for improving the anti-cancer effects of MET inhibitors in KRAS-mutated colon cancer (56). KRAS-targeted therapy has not been successfully developed previously and, therefore, these findings are promising.

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**Figure 1.** Flow diagram of the steps of phenotypic library screening with a genomic library for identifying cancer drug target genes. (A) Step 1: Loss of function, which is obtained by RNAi-mediated gene knockdown or Cas9-mediated gene knockout in cells. (B) Step 2: Phenotypic screen. Cells are subjected to various assays with different selection pressures including: 1, viability; 2, synthetic lethal; 3, viability under drug; 4, invasion/migration; 5, anoikis-resistance; 6, resistance to oncogene-induced senescence; 7, cancer stemness; and 8, tumor growth in vivo. (C) Step 3: Quantifying shRNA or sgRNA. DNA is extracted from harvested cells. Abundance of each shRNA or sgRNA is quantified using next-generation sequencing. (D) Step 4: Data analysis. Data are analyzed to generate ranked lists of promising cancer drug target genes. shRNA, short hairpin RNA; sgRNA, single-guide RNA.
| Authors, year       | Type of library     | Size of library | Cancer type                                      | Drug(s)                        | Identified genes or the pathways                                                                 |
|--------------------|---------------------|-----------------|-------------------------------------------------|--------------------------------|--------------------------------------------------------------------------------------------------|
| Bartz et al, 2006  | Pooled shRNA        | 20,000 genes    | Non-small cell lung, cervical and ovarian       | Cisplatin                      | BRCA1, BRCA2, TP53                                                                              |
| Whitehurst et al, 2007 | Arrayed RNA oligos  | 21,127 genes    | Non-small cell lung                             | Paclitaxel                     | ACRBP, ATP6V0D2, FGD4, HS6ST2, PSMA6, TUBGCP2, MCL1                                               |
| Lin et al, 2007    | Arrayed RNA oligos  | 4,000 druggable genes | Small cell lung                                | ABT-737                        | CARD11                                                                                           |
| Lam et al, 2008    | pooled shRNA        | 500 kinase genes | Diffuse large B-cell lymphoma                   | IKKβ inhibitors                |                                                                                                  |
| Xu et al, 2011     | Arrayed RNA oligos  | 22,000 genes    | Cervical                                        | Cytotoxic nucleoside analog 2', 2'-difluorodeoxyuridine and SRSF3 SFPQ |                                                                                                  |
| Guerreiro et al, 2011 | Arrayed RNA oligos  | 719 kinase genes | Medulloblastoma                                 | Cisplatin                      | ATR, LYK5, MPP2, PIK3CG, PIK4CA, WNK4                                                             |
| Liu-Sullivan et al, 2011 | Arrayed RNA oligos  | 1,657 genes     | Non-small cell lung                             | GSK461364A (PLK1 inhibitor)    | 97 genes                                                                                         |
| Prahallad et al, 2012 | Pooled shRNA        | 518 kinase and 17 kinase-related genes | Colorectal, prostate and thyroid                | Vemurafenib                    | EGFR                                                                                             |
| Fredebohm et al, 2013 | Pooled shRNA        | 1,000 genes     | Pancreatic                                      | Gemcitabine                    | RAD17                                                                                             |
| Milosevic et al, 2013 | Pooled shRNA        | 779 kinase genes | Pancreatic                                      | Erlotinib                      | RP56KA2                                                                                           |
| Wetterskog et al, 2014 | Arrayed RNAi        | 369 genes        | ERBB2-amplified breast                          | Lapatinib                      | RAB34, TP53INP1, RAC1, ATP6C1V1, C11ORF73, MLLT6, NIBP (TRAPPC9), NUF1P, PROCA1, RAB7L1, RAD21, SCR2 and SPOP |
| MacKay et al, 2014 | Arrayed RNA oligos  | 1,067 genes     | Osteosarcoma                                    | Cisplatin                      | HOIP                                                                                              |
| Maruyama et al, 2014 | Pooled shRNA        | 10,000 shRNAs   | Prostate                                        | Bicalutamide                   | RPL31                                                                                             |
| Sudo et al, 2015   | Pooled shRNA        | 16,000 genes    | Non-small cell lung                             | gefitinib                      | PRKCSH and genes involved in the CD27 signaling cascade                                           |
| Prahallad et al, 2015 | Pooled shRNA        | 298 phosphatases or phosphatase-related genes | Colorectal                                     | Vemurafenib                    | PTPN11                                                                                             |
| Kobayashi et al, 2015 | Pooled shRNA        | ~15,000 genes   | Cervical, colorectal and non-small cell lung    | 2-deoxyglucose (2DG) (glycolytic inhibitor) | COPB1, ARCN                                                                                       |
| Yamaguchi et al, 2016 | Pooled shRNA        | 2,924 genes     | Head and neck squamous cell carcinomas          | Rapamycin                      | Genes involved in the ERK pathway                                                                 |
Immune therapy using immune checkpoint inhibitors provides significant clinical benefit to patients with various types of cancer, including melanoma, lymphoma, and lung cancer (57). However, intrinsic or acquired resistance inevitably occurs, limiting the clinical benefits (49). Using genome-wide CRISPR-Cas9 or siRNA libraries, two studies identified PLN (encoding the apelin receptor) and C-C motif chemokine receptor 9 as genes that may cause resistance to immune checkpoint inhibitors (58,59).

Invasion and migration. Metastasis is significantly associated with a poor patient prognosis, and patients with metastatic cancer exhibit poor survival outcomes (60). Metastasis comprises several sequential steps: i) Migration from a primary site; ii) intravasation; iii) passage by blood flow; iv) extravasation; v) and final settlement at distant sites. To complete this process, cancer cells must acquire the ability to invade and migrate and cancer cells exhibit these oncogenic properties. Previous studies demonstrated that epithelial-mesenchymal transition (EMT) significantly contributes to metastasis in cancer cells (61,62). EMT, and its reverse phenomenon MET, were initially identified during embryonic development, in which embryonic cells transform into terminally differentiated, specialized cells via several cycles of EMT and MET (61). A number of studies suggest a central role of EMT in metastasis (63‑65). Previous studies have identified target genes for inhibiting migration and/or invasion ability of cancer cells through library screening. Pavan et al (66) developed a system combining RNAi library screening with a microscopy-based high-throughput quantitative analysis to identify a signaling pathway contributing to EMT in breast cancer. The group identified 59 genes whose inhibition suppressed transforming growth factor β-induced EMT in immortalized epithelial normal murine mammary gland cells. In addition, Pavan et al (66) focused on MEK5 and ERK5 belonging to the same signaling pathway and demonstrated the potential of targeting MEK5 and ERK5 as an anti-metastatic mechanism.

Another study used migration ability as a phenotype for functional screening, identifying genes contributing to migration in glioblastoma, a highly invasive cancer (67). The authors performed a genome-wide RNAi screening in glioblastoma cells with a functional selection of cells able to migrate through Matrigel, identifying two genes [KH-type splicing regulatory protein (KHSRP) and host cell factor C1 (HCFC1)] as targets of invasion-suppressing therapeutics for glioblastoma.

Resistance to anoikis: Anchorage-independent growth. Upon detachment from the extracellular matrix or neighboring cells, normal epithelial cells undergo a type of apoptosis called anoikis (68). Anoikis prevents normal epithelial cells from colonizing at different organ sites, thereby maintaining the integrity of the body (68). Most cancer cells acquire resistance to anoikis, which is called anchorage-independent growth (AIG). The ability of AIG allows cancer cells to metastasize to different organs and is considered a hallmark of cancer cells (64). Several different molecular mechanisms underlying AIG have been identified, including the induction of intrinsic and extrinsic anti-apoptotic signaling, often triggered by changes in the expression patterns of integrin family members (68,69). In addition, previous studies have

| Authors, year | Type of library | Cancer type    | Drug(s) | Identified genes or the pathways (Refs.) |
|---------------|----------------|---------------|---------|----------------------------------------|
| Yamanoi et al., 2016 | Pooled shRNA | Ovarian | Cisplatin | ABHD2, DCC, SLC20A, SPRY3, GSK3 (111) |
| Kurata et al., 2016 | Pooled CRISPR | Acute myeloid leukemia | Ara-C | DCK, SLC29A (53) |
| Hou et al., 2017 | Pooled CRISPR | Acute myeloid leukemia | FLT3 inhibitor | SPRY3, GSK3 (54) |
| Sun et al., 2018 | Pooled CRISPR | Hepatocellular carcinoma | Sorafenib | MEK inhibitors (55) |
| Sustic et al., 2018 | Pooled CRISPR | KRAS-mutant colon | Oxaliplatin | The ERK5-MEK-JUN pathway (56) |
| Combes et al., 2019 | CRISPR, clustered regularly interspaced short palindromic repeats (CRISPR-CAS9) | KRAS-mutant colon | Oxaliplatin | ATR (112) |
| RNAi, RNA interference | CRISPR-CAS9, clustered regularly interspaced short palindromic repeats (CRISPR-CAS9) | Colorectal | Oxaliplatin | ATR (112) |

RNAi, RNA interference; CRISPR, clustered regularly interspaced short palindromic repeats; siRNA, short hairpin RNA; sgRNA, single-guide RNA.
demonstrated the role of EMT in AIG (68,70); however, the underlying molecular mechanisms of AIG are yet to be elucidated.

Eskiocak et al (71) used the immortalized untransformed colon epithelial cell line HCEC as a model system to evaluate the effects of shRNA-mediated knockdown of selected genes on AIG. The effects of the knockdown of 151 candidate cancer genes (CAN-genes), which have been identified as genes most likely to be drivers in breast and colorectal cancers, via a comprehensive statistical and bioinformatic analysis (72), were evaluated and revealed that CAN-genes are enriched in AIG suppressors. In addition, Simpson et al conducted a genome-wide shRNA screening to identify anoikis-resistant genes by culturing immortalized prostate and nasopharyngeal untransformed cell lines in a suspension condition, which identified α/β hydrolase domain containing 4 (ABHD4) as a promising target for inducing anoikis (73).

Resistance to oncogene-induced senescence (OIS). Activation of certain types of oncogenes, such as mutant KRAS and BRAF, causes normal cells to undergo senescence (74,75). This type of senescence is stress-induced and is termed OIS (74,75). OIS functions as a barrier to carcinogenesis initiated by normal cells, whereas senescence-associated secretory phenotype (SASP) is a carcinogenesis-promoting aspect of OIS (76). OIS was discovered by Serrano et al (77), who demonstrated that oncogenic ras induces premature senescence in experimental cell cultures. Subsequently, the occurrence of OIS in human disease was demonstrated in a developmental process of melanoma. Two studies revealed that OIS prevents benign melanocytic nevi, presumed to be the origin of melanoma, from transforming to overt melanoma (78,79). These studies demonstrated that proliferation of nevi cells is suppressed at very low levels despite harboring the highly oncogenic mutation BRAFV600. Studies have also revealed that BRAFV600-induced OIS is associated with p16INK4A upregulation (78,79); however, other unidentified changes may also be involved because of the complex mechanisms governing senescence (80). Therefore, several studies have attempted to identify genes that promote cells to bypass senescence induced by oncogenic RAS or BRAF. Vicent et al (81) performed RNAi screening to identify genes that facilitate bypassing ras-induced OIS in mouse models, reporting that Wt1 transcription factor (Wt1) is an OIS-bypassing gene using both in vitro and in vivo models. Vicent et al (81) also demonstrated WTI to be an independent prognostic factor in patients with KRAS-mutated lung cancer. Another study performed a near-genome-wide screening (~15,000 genes) to identify regulators of BRAFV600-induced senescence and identified RASSF as an OIS suppressor (82). Some screenings searching for OIS-bypassing genes used primary normal culture, and immortalized untransformed normal cell lines as model systems (82,83) because these cells are more prone to exhibit OIS; primarily due to their intact senescent machinery. However, the ability of cancer cells to undergo OIS may be impaired because of alterations in genes involved in senescence (74). Therefore, the applicability of the identified OIS-bypassing genes needs to be validated in multiple human cancer cell lines before exploring their usefulness as drug targets.

Cancer stemness. The cancer stem cell (CSC) theory hypothesizes that CSCs have the ability to self-renew and to differentiate into phenotypically diverse cancer cells (84). Although the CSC concept has not been demonstrated, accumulating evidence suggests that a number of types of cancer harbor CSCs (84,85). Notably, CSCs are hypothesized to be resistant to chemotherapy and irradiation (84). Therefore, the development of CSC-targeted therapeutics is attracting attention because of its potential to eradicate cancer cells. A functional library screening based on the sphere-forming ability of breast cancer cell lines identified ATG4 as a promotor of the breast CSC-like phenotype (86). However, the usefulness of a sphere-formation assay for evaluating the self-renewal capacity is based on the assumption that the assay developed for normal neural stem cells can be accurately used for CSCs. Therefore, validation of genes identified as cancer stemness genes by other assays, such as a transplantation assays and lineage-tracing approaches, are required.

Genomic Instability. A phenotype of genomic instability facilitates diverse oncogenic properties because it causes numerous mutations resulting from the activation of oncogenic genes or inactivation of tumor suppressive genes (87). A previous study performed a genome-wide RNAi screen to identify the pathways and specific genes mediating genomic stability (88). A screen using elevation of γH2AX variant histone (H2AX; a marker of double strand DNA damage) as an indicator for detecting DNA damage was conducted in HeLa cancer cells, identifying genes involved in DNA replication, checkpoint activation and DNA repair. The identified genes included TIMELESS and TIPIN encoding proteins that form a complex, leading to activation of the replication checkpoint. The identified genes may serve as promising drug targets to restore genomic stability in cancer cells (88).

Tumor growth in vivo. Tumor growth in vivo represents a more accurate screening method because it accounts for several aspects of real tumor growth, including 3-D condition, requirements for angiogenesis and the microenvironment (89).

One critical issue of in vivo pooled library screening is the difficulty in ensuring appropriate representation of the entire library in the initial population inoculated into model animals (89). There is a limitation to the maximum number of cells that can be inoculated, which limits the size and complexity of the library. Notably, the minimum cell numbers required for each shRNA differ significantly, depending on whether tumor suppressor genes or oncogenic genes are targeted for screening (89). In the case of oncogenic gene-targeted screening, low library representation tends to result in false-positive results, so instead of using genome-wide libraries, researchers used libraries focused on specific types of genes in order to identify oncogenic genes. For example, Singh et al (90) used an shRNA library of 150 genes associated with brain metastasis to conduct a library screening consisting of both in vivo (intracranial injection) and in vitro (tumor sphere-forming assay) assays to identify metastasis-promoting genes. The group successfully identified SPARC (osteonectin), cwcv and kazal like domains proteoglycan 1 (SPOCK1) and twist family bHLH transcription factor 2 (TWIST2) as
regulators of brain metastasis-initiating cells. In addition, most studies using in vivo genome-wide or near-genome-wide RNAi models discovered tumor-suppressive genes (91,92).

3. Conclusions and future perspectives

Shortly after RNAi technology for gene knockdown was developed in the laboratory, attempts to conduct large-scale functional screenings with RNAi were initiated (93). In addition, a gene knockout technique, CRISPR-Cas9 was also introduced for laboratory use (94). For >10 years, researchers have extensively conducted functional genomic screening to identify better targets and to develop new therapeutics for cancer. The present paper reviewed and summarized knowledge obtained by these studies, which has the potential to be used for drug development. Nevertheless, breakthroughs that can be immediately translated into clinical use are yet to be made. In particular, despite many reported studies, KRAS synthetic genes that have been reproducibly confirmed have not been successfully identified; therefore, development of KRAS-synthetic lethal drugs has not been successful.

Project DRIVE suggested that no single synthetic lethal genes for KRAS exist. However, there may be certain strategies potentially enabling the identification of true KRAS synthetic genes; for example, one approach may be using more realistic modeling systems to evaluate malignant phenotypes. Such models may include a 3-D culture of cell lines and patient-derived xenografts (95,96), although such models are usually difficult to manage for large-scale screening. Due to the large heterogeneity in coexisting genomic alterations among KRAS-mutated tumors, studies using cancer cells may suffer from the presence of high background of noise during screening. Therefore, focus is needed on cancer cells which have higher similarities in harbored genetic alterations in addition to mutant KRAS.

An improvement in consistency of identified genes from a genome-wide screen has been revealed in CRISPR-Cas9 knockout compared with shRNA techniques (28). However, pharmacological inhibition of gene function with compounds is usually incomplete; thus, target genes identified through partial knockdown with RNAi represent improved targets. Therefore, results from CRISPR-Cas9 and RNAi screens need to be regarded as complementary.

In conclusion, advances in the technology of gene silencing and next generation sequencing have enabled researchers to conduct large-scale high-throughput phenotypic screenings, resulting in the identification of numerous potential novel drug targets for cancer. However, there are several issues, such as low reproducibility in the identified genes (40). Thus, substantial effort is required to adequately address these problems in order to identify novel cancer drug target genes.

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Author's contributions

MS designed the review, researched the literature and wrote the manuscript.

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Competing interests

The author declares that he has no competing interests.

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