VIA MEDICA

REVIEW ARTICLE

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Use of next-generation sequencing in daily routine practice

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Translation: dr n. med. Dariusz Stencel
Oncology in Clinical Practice
DOI: 10.5603/2023.0001

ISSN 2450–1654
e-ISSN 2450–6478

ABSTRACT

Developments in molecular diagnosis and implementation of mutation-driven targeted therapy marked a milestone in cancer treatment. Next-generation sequencing allows sequencing of a high number of nucleotides in a short time and from a limited quantity of pathology or cytology specimens. This is a review of actual indications, utility of next-generation sequencing, and availability of targeted therapies in different neoplasms. We present the European Society for Medical Oncology Precision Medicine Working Group recommendations for tumor multigene sequencing use with the Scale for Clinical Actionability of molecular Targets ranking determined for each alteration.

Key words: next-generation sequencing, targeted therapy, experts recomendations

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Introduction

Next-generation sequencing (NGS) is currently the most advanced method of molecular biology used in genetic diagnostics. The main advantage of NGS is its ability to evaluate many genetic markers and classes of mutations during one test and from one tissue or cell sample. A growing understanding of the underlying molecular biology of cancer accelerates the development of targeted therapy. However, the availability of drugs targeting these genetic abnormalities varies between solid tumors. We present a review of current indications for NGS in daily clinical practice, taking into account the recommendations of the European Society of Medical Oncology (ESMO) Precision Medicine Working Group.

Methodology of next-generation sequencing

Biological material for genetic testing should be collected after obtaining patients' written consent for diagnostic genetic testing and sent directly for pathological evaluation. Based on qualitative and quantitative assessment of tissue samples and tumor cell percentages, the pathologist evaluates if the sample is suitable for molecular testing and selects the most representative specimen. The diagnostic material is usually paraffin-embedded tissue and, alternatively, cytological preparations (cytoblocks or smears) or, in selected situations, circulating tumor DNA (ctDNA). Before the molecular analysis, a histological preparation is made from the paraffin block, which enables morpho-

Received: 21.11.2022 Accepted: 21.11.2022 Early publication date: 04.04.2023

logical verification in terms of the content and location of cancer cells in the preparation. Evaluated preparations should contain not less than 20% of tumor tissue. The amount of nucleic acids necessary for NGS analysis, depending on the test manufacturer, is on average about 200 ng of DNA/RNA. The quality of the isolated nucleic acids is crucial. Quantification should be based on measurement using a fluorometer, a device based on the fluorescence intensity of fluorescent dye binding to DNA/RNA. Quality assessment (integrity and presence of enzyme reaction inhibitors) is measured by dedicated quality tests using the polymerase chain reaction (qPCR).

Genetic abnormalities can be assessed at the RNA- and DNA-level. It should be emphasized that in the case of identifying gene fusions, NGS is currently the gold standard, evaluating genetic variations at the RNA level. The main advantages of this method for identification of gene fusions are: high sensitivity and specificity, the ability to identify many gene fusions during one test, the ability to identify fusion partners and the exact locations of breakpoints in the identified fusion partners, the ability to assess whether the identified fusion is contained in the reading frame (pathogenic variant, functional or non-functional, with no clinical relevance). In addition to pointing at mutations, small deletions/insertions, and gene fusions, it is also possible to test for microsatellite instability (MSI) and tumor mutation burden (TMB, number of mutations per 1 million base pairs of the cancer genome), as well as the homologous recombination deficiency (HRD).

In cases of identifying a rare mutation variant or fusion variant not yet reported, the results of NGS should be confirmed by another method. Sanger sequencing, a method of DNA sequencing, which can verify variants or fusion junctions in DNA is typically used to confirm changes.

The genetic test report should contain the result, its precise interpretation understandable to the clinical oncologist and pathologist, as well as the description and scope of the method used. The laboratory issuing the result should have a confirmation of the current certification of the European external quality control program for a given test. NGS results should be available within 20 working days from sample delivery.

Genetic tests must be performed using equipment with full documentation of repairs, validations, and annual inspections (Ministry of Health regulation of March 21, 2006 [1]). The laboratory must meet the requirements described in the Ministry of Health Regulation on standards for medical diagnostic and microbiology laboratories [2].

Determining the value of NGS tests in clinical practice

The indications and value of NGS tests in individual cancers were the subject of recommendations of ESMO Precision Medicine Working Group experts [3]. The indications for performing NGS in daily clinical practice were evaluated in comparison to molecular diagnostics methods currently used. Based on the analyzes performed, individual genetic disorders were classified according to the ESMO Scale for Clinical Actionability (ESCAT), depending on the availability of the appropriate drug in daily clinical practice (Tab. 1 and 2). It should be highlighted, that the cost of NGS tests is higher than the cost of simpler molecular diagnostics methods. This is especially true for indications where the availability of drugs targeting particular molecular pathways is limited.

Non-small cell lung cancer

Activating mutations in the *EGFR* gene were the first to be investigated and constituted the basis for advances in the treatment of patients with advanced non-small cell lung cancer (NSCLC) of non-squamous type [4]. For the most common activating mutations, such as deletion in exon 19 and point mutation in exon 21 (L858R), all 3 generations of tyrosine kinase inhibitors (TKI) (erlotinib, gefitinib, afatinib, dacomitinib, and osimertinib) are active. Many randomized studies have demonstrated the effectiveness of these drugs in EGFR-positive NSCLC [5–7]. Rare mutations involving exons 18–21 of the *EGFR* gene (G719X exon 18, L861Q exon 21, S768I exon 20) have been shown in several non-randomized studies to be associated with prolongation of progression-free survival (PFS) in patients

Table 1. Scale for clinical actionability of the observed genetic disorders

ESCAT Level	Definition
I	Drug has clinically proven activity in a given molecular disorder and is used in clinical practice
II	Drug activity was demonstrated in phase I and II clinical trials or retrospective analyzes of randomized controlled trials
III	Drug activity is observed in genetic disorders in another indication
IV	Potentially treatable genetic disorders observed in preclinical studies

ESCAT — ESMO Scale for Clinical Actionability

Table 2. ESMO Scale for Clinical Actionability (ESCAT) levels for selected molecular abnormalities in various cancers

Diagnosis	Genetic disorder	ESCAT level
NSCLC	*EGFR* — del19, L858R, acquired T790M exon 20, other (G719X ex18, L861Q exon 21, S768I exon 20)	I
	ALK, *MET* exon 14, *BRAF* V600E, *ROS1*, *NTRK*, *RET*	
	EGFR — exon 20 insertions	II
	MET amplification, *KRAS* G12C, *HER2*	
Prostate cancer	*BRCA 1 and 2*, MSI-H	I
	PTEN, *ATM*, *PALB2*	II
Cholangiocarcinoma	*FGFR2*, *IDH1*, *NTRK*	I
	BRAF V600E	II

NSCLC — non-small cell lung cancer

receiving afatinib and Osimertinib [8, 9]. In the group of patients with an *EGFR* gene exon 20 insertion, mobocertinib was shown to be effective in terms of PFS [10]. The drug received Food and Drug Administration (FDA) approval for the treatment of NSCLC patients with exon 20 insertion after failure of platinum-based chemotherapy. Amivantamab was granted European marketing authorization for this indication. In a phase II study, 40% objective responses and a median time to disease progression of 8.3 months were observed among patients treated with amivantamab after chemotherapy failure [11].

In patients with disease progression on first- or second-generation tyrosine kinase inhibitors, the presence of the T790M resistance mutation in exon 20 should always be assessed. Confirmation of the presence of this disorder is an indication for osimertinib treatment [12].

Another molecular disorder assessed during diagnostics of advanced non-squamous NSCLC is rearrangement in the *ALK* gene. Many randomized studies have confirmed the effectiveness of ALK tyrosine kinase inhibitors in patients with confirmed *ALK* gene rearrangement [13–16]. Three generations of ALK pathway inhibitors are currently used in clinical practice — crizotinib, alectinib, brigatinib, ceritinib, and lorlatinib.

In patients with advanced NSCLC with *MET* gene exon 14 skipping mutation (METex14), the efficacy of tepotinib and capmatinib was confirmed based on a significantly increased objective response rate (ORR) [17, 18]. Both drugs have received European registration for use in patients with METex14 after failure of previous immunotherapy and/or platinum-based chemotherapy.

The V600E mutation in the *BRAF* gene occurs in 2% of patients with non-squamous NSCLC. The combination of dabrafenib and trametinib has been shown to be effective in patients with this disorder [19].

In patients with *NTRK* gene fusion, the efficacy of entrectinib was confirmed in phase I and II studies (STARTRK-1, STARTRK-2), and the drug was registered by the European Medicine Agency (EMA) [20]. Entrectinib is also active in patients with *ROS1* gene fusion.

The G12C mutation in the *KRAS* gene occurs in approximately 12% of patients with non-squamous NSCLC. The effectiveness of sotorasib in the treatment of patients with NSCLC with the G12C mutation of the *KRAS* gene after failure of chemotherapy and immunotherapy was evaluated in the CodeBreaK100 [21] and Code-Break200 studies, which compared the efficacy of the drug with docetaxel. The approximately 18-month follow-up confirmed improvement in PFS (HR = 0.66; 95% CI 0.51–0.86; p = 0.002) and ORR (28.1 *vs.* 13.2%) after sotorasib treatment compared to docetaxel [22]. Another drug active in this group of patients is adagrasib, which was evaluated in the phase I/II KRYSTAL study in the population of patients with the *KRAS* gene mutation after failure of chemotherapy and immunotherapy. The primary endpoint was the objective response rate, which was 42.9%; the median time to disease progression was 6.5 months, and overall survival was 11.7 months [23]. Selpercatinib, a small-molecule RET kinase inhibitor showed efficacy in a phase I/II study, in the form of an increased objective response rate (ORR) in patients with NSCLC with *RET* gene fusions [24]. Mutations in the human epidermal growth factor receptor 2 (HER2), which is a member of the ErbB family of receptor tyrosine kinases, occur in about 3% of patients with NSCLC. In patients with HER2-positive NSCLC after chemotherapy failure, the efficacy of the immunoconjugate trastuzumab derukstecan was confirmed. The objective response rate, which was the primary endpoint in a phase II study, was 55%, and the mean time to disease progression was 8.2 months [25].

Taking into account the increasing number of molecular disorders assessed when qualifying patients with advanced non-squamous NSCLC for treatment and the possibility of using appropriate molecularly targeted therapy in daily clinical practice, it seems reasonable to use NGS, which is in line with the ESMO recommendations for NGS testing in patients with non-squamous lung cancer to detect treatable ESCAT Level I molecular changes. If appropriate drugs are available, NGS should also capture a broader gene profile.

Urogenital neoplasms

Undoubtedly advances in the treatment of patients with urinary tract neoplasms result, among others, from the introduction of more and more accurate diagnostic methods and several new therapeutic strategies into clinical practice. The latter include application of the so-called modern hormonal drugs at various stages of treatment in patients with metastatic prostate cancer [26], targeted therapies and immunocompetent drugs in patients with renal cell carcinoma (RCC) [27], as well as immunotherapy, antibody-cytostatic conjugates, and targeted therapies in patients with urothelial cancer [28]. However, it should be remembered that not all patients benefit from treatment, which may additionally be associated with significant toxicity, therefore, it is extremely important to search for biomarkers that allow for treatment personalization.

Castration-resistant prostate cancer

Molecular tests indicate that approximately 30% of patients with castration-resistant prostate cancer (CRPC) have abnormalities in DNA repair genes. Germline mutations are present in about 12% of patients, and the frequency of somatic mutations increases with disease progression [29]. Therefore, the efficacy of poly (ADP-ribose) polymerase (PARP) inhibitors in this indication was assessed. Based on the PROfound study, olaparib was approved [26]. It should be emphasized that the EMA indication [treatment of patients with metastatic castration-resistant prostate cancer (mCRPC) with confirmed germline or somatic mutation in *BRCA1* or *BRCA2* genes] and the FDA-registered indication [mCRPC with the presence of germinal or somatic mutations in homologous recombination repair (HRR) genes] are different. Another PARP inhibitor, rucaparib, received FDA accelerated approval in patients with mCRPC with a mutation in the *BRCA1/2* gene after previous use of new hormonal drugs and docetaxel [27]. The drug is not registered in this indication by the EMA.

Combinations of PARP inhibitors with new hormone therapy (e.g. abiraterone or enzalutamide) may also be a therapeutic option in patients with mCRPC. The PROpel study evaluated the combination of abiraterone acetate with olaparib compared to abiraterone acetate with placebo — in the general population, median radiographic PFS (rPFS) was longer by more than 8 months (HR = 0.66; 95% CI 0.54–0.81) [30]. In a subgroup analysis, a greater benefit was found in patients with mutations in HRR genes. OS data is immature. In the MAGNITUDE study, the benefit of combining niraparib with abiraterone acetate was evaluated in patients with mutations in HRR genes, and it was greater in patients with mutations in *BRCA1* and *BRCA2* genes [31]. In countries where PARP inhibitors can be used in this indication, NGS is recommended in patients with advanced prostate cancer (recommendation I).

Urothelial carcinoma

Patients with metastatic urothelial carcinoma (mUC) continue to have a poor prognosis. Platinum-based chemotherapy (preferably cisplatin) is the primary treatment, which allows for obtaining short-term disease control in the majority of patients (about 20% of patients show primary resistance to treatment) [32]. Prolongation of OS is possible after use of maintenance immunotherapy [33].

Erdafitinib, a fibroblast growth factor receptor (FGFR) tyrosine kinase inhibitor, is a targeted therapy registered by the FDA for the treatment of patients with mUC. The FGF pathway is associated with the proliferation, migration, and invasiveness of cancer cells. Mutations or rearrangements are found in about 20% of patients with mUC, and significantly more often in urothelial carcinoma of the upper urinary tract. In a pivotal study, the use of erdafitinib in patients with the aforementioned disorders previously receiving systemic treatment resulted in an objective response rate of approximately 40% [34]. RT-PCR is the recommended test for routine diagnostics..

Renal cell carcinoma

Systemic treatment of patients with renal cell carcinoma (RCC) has progressed with the use of targeted drugs (multikinase inhibitors) and immune checkpoint inhibitors (ICIs) (alone or in combination) as well as sequential treatment. Molecular predictors for these therapies have not yet been determined. It is worth noting, however, that approximately 13% of patients with papillary carcinoma have overexpression of MET kinase. Based on the results of the SWOG1500 (PAPMET) study, in the treatment of RCC patients with this disorder, cabozantinib is preferred due to its activity against the HGF/MET pathway [35]. It is worth noting that in the SAVOIR study savolitinib, an MET inhibitor, was not significantly more effective compared to sunitinib and is not registered in the treatment of patients with RCC [36]. There are no ESCAT recommendations regarding genetic diagnostics in RCC patients.

Breast cancer

Due to the availability of routine diagnostic methods (RT-PCR, immunohistochemistry), which enable qualification for targeted therapy, NGS with the use of tumor sample is not recommended in routine clinical practice in breast cancer patients [3]. On the other hand,

assessment of germline mutations in *BRCA1/2* genes using the NGS method is already a common diagnostic standard, aimed at qualifying patients for targeted therapies or modifying standard treatment regimens.

Ovarian cancer

Due to the greater sensitivity to PARP inhibitors in patients with ovarian cancer with the *BRCA1/2* gene mutation, the ESCAT recommendations allow for the routine use of multi-gene NGS panels to identify this population [3]. The NGS study plays an important role in this case because it allows not only for determination of the status of *BRCA1/2* genes but also the so-called HRD genomic signature. In addition, it should be highlighted that the benefit of PARP inhibitors in patients with ovarian cancer is probably independent of the *BRCA1/2* genes status, which reduces the practical advantages of using NGS [37].

Gastrointestinal (GI) neoplasms

For almost two decades, targeted therapies have been an important element in the treatment of some GI malignancies [38]. Initially, it concerned selected cancers (colorectal cancer or hepatocellular carcinoma), but emerging new molecular targets expanded the range of indications. The need to detect appropriate biomarkers, necessary to benefit from the use of some drugs, has led to spreading of comprehensive molecular diagnostics (including NGS). At the same time, the routine use of polygenic panels in clinical practice is limited to some patients with gastrointestinal cancers.

Colon cancer

Modern treatment of patients with metastatic colorectal (CRC) or rectal cancer is based on the use of biomarkers. Detection of *hotspot* mutations in *KRAS*/*NRAS* genes determines resistance to anti-EGFR antibodies, preventing their use in this patient population [39]. In turn, the detection of the $BRAF^{V600E}$ mutation, which is an important prognostic factor, makes it possible to use the combination of encorafenib with cetuximab in the second line of systemic treatment [40]. Diagnostics of *KRAS*/*NRAS* and *BRAF* genes status are based on the PCR method and are usually performed sequentially due to the extremely rare coexistence of *KRAS*/*NRAS* and $BRAF^{V600E}$ mutations. The high-level microsatellite instability (MSI-H) is a biomarker playing an increasingly important role as a selection factor for immunotherapy in the first and subsequent treatment lines [41]. Microsatellite instability status is routinely assessed by immunohistochemistry (ICH) or PCR. The last of the unambiguously recognized biomarkers in this population are *NTRK* fusions although it should be emphasized that the frequency of their occurrence in patients with metastatic colorectal cancer is very low (about 0.5%). There is currently no clear consensus on how to detect *NTRK* fusions. It is often suggested to use immunohistochemistry as a screening method and to use molecular biology methods only in patients with a positive IHC result [42].

Another biomarker of potentially significant importance are disorders in the *HER2* gene (mainly amplifications), as there are more and more data on the effectiveness of HER2 receptor blockade [43]. The primary diagnostic method, in this case, is IHC with the possible use of fluorescent in situ hybridization (FISH) in ambiguous situations. Further biomarkers may be used in the future (e.g. *PIK3CA* mutation, *RET* and *ALK* fusions, or *MET* amplifications), but given the lack of consensus regarding treatment when such disorders are detected, they should be considered the domain of clinical trials.

The presence of numerous potential biomarkers of practical clinical significance would support the routine use of NGS in patients with metastatic colorectal or rectal cancer. An additional benefit could be the acceleration of the diagnostic process, which is already multi-stage and includes at least the determination of the status of *KRAS*/*NRAS* genes with a possible sequential assessment of the *BRAF* gene, and an independent MSI assessment. Nevertheless, the current recommendations do not suggest a routine replacement of standard PCR with the NGS method in colorectal cancer patients (note — NGS may be considered unless it is associated with significantly higher costs). The potential benefit of using multi-gene NGS panels would mainly concern the identification of patients with *HER2* gene amplification and routine assessment of NTRK fusion [3].

Bile duct cancer

Bile duct cancers, also called cholangiocarcinomas, are a diverse group of cancers that are characterized by significant molecular differences. The difference depends on the level of the bile ducts from which the cancer originates. Targeted therapies are currently most useful in intrahepatic cholangiocarcinomas, where *FGFR2* gene fusions (10–15% of patients) and *IDH1* gene mutations (up to 20% of patients) are detected more often than in other cholangiocarcinomas. In the presence of *FGFR2* fusions, the use of FGFR inhibitors (e.g. pemigatinib or infigratinib) allows for high response rates, exceeding the values obtained with standard chemotherapy [44]. From the perspective of molecular biology, the detection of *FGFR2* gene fusions, especially with rare or novel partners, is difficult and requires the use of NGS or modified PCR [45].

In terms of the clinical significance of the *IDH1* gene mutation, there is evidence of the effectiveness of ivosidenib, whose use improved PFS and OS [46]. There is currently no consensus on the optimal method for detecting *IDH1* mutations, and possible strategies include the screening use of IHC or the baseline use of PCR or NGS [47]. These molecular abnormalities concern almost exclusively intrahepatic cholangiocarcinomas, but other subtypes of cholangiocarcinoma are also characterized by the possibility of the presence of significant biomarkers. The emerging reports on the effectiveness of targeted therapies in patients with the *BRAF*^{V600E} mutation or *HER2* gene amplification are noteworthy [48, 49]. As in the case of other gastrointestinal cancers, the possibility of detecting MSI-H and *NTRK* fusion should be considered [42, 50]. Other regularly occurring molecular disorders (e.g. *BRCA1/2* and *PIK3CA* mutations or *MET gene* amplifications) do not currently translate into additional treatment options and are only relevant in clinical trials.

Due to the nature of the detected molecular disorders, the use of NGS is an option for routine diagnostics in patients with cholangiocarcinoma, which results from the specific nature of the most common biomarkers (*FGFR2* fusions and *IDH1* mutations), for which NGS is considered one of the reference methods [3]. However, attention should be paid to the high costs associated with the routine use of multi-gene NGS panels and the alternative possibility of using dedicated NGS panels, covering only selected biomarkers.

Pancreatic cancer

The possibilities of targeted therapy in patients diagnosed with advanced pancreatic adenocarcinoma remain scarce and concern mainly patients with confirmed germline *BRCA1/2* mutations in whom maintenance treatment with PARP inhibitors may be considered after initial platinum-based chemotherapy [51]. Possible detection of the *BRCA1/2* mutation in multi-gene NGS panels requires confirmation of the germinal nature of the mutation before the possible use of PARP inhibitors. Taking into account the relatively rare occurrence of other genetic disorders qualifying for targeted therapy (high TMB, *KRAS G12C* mutation, *NTRK* fusions), the routine use of multi-gene NGS panels in patients with pancreatic cancer is not recommended [3].

Other GI malignancies

Despite the widespread use of targeted therapies in advanced cancers originating in the gastrointestinal system, we do not have predictive biomarkers for most of the therapies used. Therapies that are agnostic to the origin of cancer (e.g. immunotherapy in the case of MSI-H or NTRK inhibitors in the case of *NTRK* fusions) have brought some change in recent years [42, 50]. The list of such agnostic therapies is likely to get longer. Unfortunately, some biomarkers will elude unambiguous assessment, for example TMB, whose determination in gastrointestinal cancers is currently recommended only in the case of neuroendocrine tumors [3]. Therefore, taking into account alternative methods of MSI-H and *NTRK* fusion assessment, routine NGS in GI malignancies, other than those described above, is not recommended. However, it should be emphasized that NGS may be indicated as a screening method in centers conducting scientific research when qualifying patients for appropriate clinical trials.

Other neoplasms

Diagnostics using NGS may be considered in the absence of other diagnostic methods and access to treatment for patients with specific genetic disorders. An example is tropomyosin inhibitors in patients with *NTRK* rearrangements (found in patients with secretory carcinomas of the salivary glands and breasts, thyroid cancers, and sarcomas) [3].

Conclusions

The advantage of NGS is its ability to evaluate multiple genetic markers from one tissue or cell sample. In indications where it is possible to use specific groups of targeted therapies depending on the present genetic disorder, the NGS test is the recommended diagnostic option. Taking into account the available therapeutic methods, the highest value in clinical practice is to perform NGS in advanced NSCLC, prostate cancers, and biliary tract cancers. The discussion concerns the size of the gene panel covered by NGS. In centers conducting scientific research, including basic research and phase I/II clinical trials, the NGS method covering a wide panel of gene disorders is indicated as a screening method during qualification of patients for appropriate clinical trials.

Author contributions

All authors: conceptualization, methodology, writing.

Funding

No funding.

Acknowledgments

None.

Conflict of interest

Adam Płużański — fees from AstraZeneca, MSD, BMS, Takeda, Jansen, Amgen; travel grants from BMS, MSD.

Maciej Kawecki — fees/lecture fees from Celgene, Novartis, Pfizer, Amgen, Roche, Merck, Novocure, Servier, and MSD; travel grants from Amgen, Novartis, Bristol-Myers Squibb, Pfizer, Roche, TechDow, MSD, Sanofi, Merck, and Angelini Pharma; clinical trials for LSK, MSD, Bristol-Myers Squibb, Beigene, AstraZeneca, MacroGenomics, Roche, Novocure, Junshi Bioscience, CStone Pharmaceuticals, and Astellas.

Maciej Krzakowski — participation in advisory boards for AstraZeneca, BMS, Roche, Boehringer-Ingelheim, and Takeda; travel grants from AstraZeneca, Roche.

Kinga Winiarczyk — declares no conflict of interest.

Andrzej Tysarowski — participation in advisory boards for Boehringer Ingelheim, Bayer, Astra Zeneca, Pfizer, BMS, Novartis, Roche, and Amgen.

Jakub Kucharz — research grants from Novartis; lecture fees from BMS, IPSEN, Pfizer, Novartis, MSD, Astellas, Janssen-Cilag, and Merck; participation in advisory boards for IPSEN, Pfizer, MSD, BMS, Astellas, and Merck; travel grants from BMS, IPSEN, Pfizer, Novartis, and MSD.

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