Phosphorylated Ataxia Telangiectasia Mutated (pATM) Enzyme-Linked Immunosorbent Assay (ELISA) for Predicting Radiation Induces Normal Tissue Toxicity in Radiotherapy Patients: A Systematic Review

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ABSTRACT
Background: An adverse normal tissue response, such as normal tissue toxicity (NTT), is present in radiotherapy (RT) patients and can limit the effectiveness of the RT treatment. Identifying patients with adverse tissue responses before RT had clinical benefits and individual radiosensitivity (IRS) is considered an important factor in NTT incidences. Therefore, this systematic study aimed to determine the possibility of using phosphorylated Ataxia Telangiectasia Mutated (pATM) Enzyme-Linked Immunosorbent Assay (ELISA) to predict NTT in RT patients.

Methods: A comprehensive data search was conducted in three electronic databases, namely PUBMED CENTRAL, ScienceDirect, and SCOPUS. The quality of relevant publications was independently evaluated using the PICO (participants, intervention/exposure, comparison, and outcome) approach.

Results: A total of 47 articles were retrieved, 41 of which were assessed based on the titles and abstracts. Furthermore, 39 articles were excluded, and 2 were included in this study.

Conclusions: The phosphorylated ATM ELISA on lymphocytes showed promising results for IRS prediction in RT patients. However, these assumptions should be validated on a larger RT patient cohort.

INTRODUCTION
Radiation treatment is beneficial for more than half of all cancer patients. However, even when there are no mistakes in the delivery of the dose, complications and adverse effects can occur in a small percentage of individuals. The levels of normal tissue radiation damage vary depending on the treatment site, irradiation mode, and prescription dose [1]. Patients receiving radiotherapy (RT) experience adverse tissue response events in 5–20% of cases, which may restrict the use of the RT treatment [2–4]. Therefore, identifying patients with adverse tissue reactions before RT is implemented might have beneficial clinical effects [4]. The frequency and severity of radiation-induced normal tissue toxicity (NTT) are multifactorial and arise from a complex interaction between patients’ tumors and treatment-related variables. Early toxicity occurs during the first three months of RT, and it is difficult to predict. Concurrent chemotherapy improves a tumor’s response to radiation through several mechanisms, including increased cell sensitivity to radiation and DNA damage and repair.
Therefore, this form of chemotherapy enhances both acute and late toxicities [5].

The intensity of NTT is determined by several factors, but individual radiosensitivity (IRS) is the dominant. Even though the term “radiosensitivity” is used internationally, it is currently debated among experts. IRS can be described as a given person’s innate capacity to show a specific toxicity profile to radiation. Meanwhile, NTT can be observed in irradiated areas, and its severity depends on IRS [4].

There has been no agreement in the literature on which severity of normal tissue response is the most important to predict. The Common Terminology Criteria for Adverse Events (CTCAE) is the most commonly used grading scale. This tool rates response on a six-point scale, namely (0) none, (1) mild, (2) moderate, (3) severe, (4) life-threatening, and (5) death.

Many biological endpoints have been investigated to predict radiosensitivity since the 1950s. An appropriate predictive endpoint should be quantitatively correlated with clinical features irrespective of tumor location, treatment dose, and RT modality. Clonogenic cell survival and cytogenetics assays are the most commonly used assays for assessing biological markers as endpoints to predict IRS. However, these predictive assays were laborious and time-consuming. Unrepaired DNA double-strand break (DSB) is also considered the primary lesion causing radiation-induced cell death. Immunofluorescence methods have revolutionized the measurement of DSB for healthy and malignant cells. Measuring unrepaired DSB requires investigation 24 hours after radiation [2].

Another approach for the IRS predictive assay is based on the nucleoshuttling of ataxia-telangiectasia mutant (ATM) kinase, a major protein of DSB repair and signaling. ATM protein, which was a critical component of the IR response, is a cytoplasmic protein translocating to the nucleus following irradiation [6]. ATM monomers facilitate the phosphorylation of H2AX histone variants (γ-H2AX), which are an early sensor of DSB identification by the nonhomologous end-joining (NHEJ) pathway.

The nucleoshuttling of ATM kinase-based reliable predictive assay for IRS was first proposed by radiobiological investigators in France [3]. A comprehensive explanation of radiation-induced ATM nucleoshuttling (RIANS) to predict IRS [7]. Among the many techniques available for determining the rate of RIANS, enzyme-linked immunosorbent assay (ELISA) is the most promising in clinical settings. Therefore, this study aims to review the use of phosphorylated ATM (pATM) ELISA for predicting IRS and NTT in RT patients.

METHODS

A complete data search was independently conducted using three electronic databases, namely PUBMED CENTRAL, ScienceDirect, and SCOPUS. The search terms employed in the literature search were “pATM,” “ELISA,” “radiosensitivity,” and “cancer” (Table 1). Relevant articles were identified by reading the references and were included after meeting the inclusion criteria (Table 2). Furthermore, the PICO (participants, intervention/exposure, comparison, and outcome) approach was used to screen the articles. Studies were evaluated after predicting the amount of NTT induced by RT (O) using pATM ELISA in RT patients as participants (P) when receiving RT treatment (I), and divided into radioresistant and radiosensitive groups (C).

RT, radiotherapy; NTT, normal tissue toxicity; CTCAE, common terminology criteria for adverse events; RTOG, radiation therapy oncology group; ELISA, enzyme-linked immunosorbent assay; pATM, phosphorylated ataxia-telangiectasia mutated; PDF, Portable Document Format; PMC, PubMed Central.

Table 1. Search strategy

<table>
<thead>
<tr>
<th>Database</th>
<th>Keywords</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>PMC</td>
<td>((pATM) AND radiosensitivity) AND cancer AND ELISA</td>
<td>31</td>
</tr>
<tr>
<td>ScienceDirect</td>
<td>pATM radiosensitivity ELISA</td>
<td>14</td>
</tr>
<tr>
<td>SCOPUS</td>
<td>(TITLE-ABS-KEY(pATM) AND TITLE-ABS-KEY(radiosensitivity) AND TITLE-ABS-KEY(ELISA))</td>
<td>2</td>
</tr>
</tbody>
</table>

Table 2. Inclusion and exclusion criteria for article selection

<table>
<thead>
<tr>
<th>Inclusion criteria</th>
<th>Exclusion criteria</th>
</tr>
</thead>
<tbody>
<tr>
<td>Participants were RT patients</td>
<td>ELISA was not used for pATM quantification</td>
</tr>
<tr>
<td>NTT was determined using CTCAE or RTOG</td>
<td>The full text could not be retrieved in PDF format</td>
</tr>
<tr>
<td>RT patients’ pATM levels were assessed</td>
<td></td>
</tr>
<tr>
<td>Articles were published during or before September 2022</td>
<td></td>
</tr>
<tr>
<td>Articles were written in English</td>
<td></td>
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</table>
RESULTS

The recommended reporting items for systematic reviews and meta-analyses (PRISMA) flowchart showed the systematic procedure used for this review (Figure 1). The comprehensive keyword search generated 31, 14, and 2 articles in PMC, ScienceDirect, and SCOPUS, respectively. Zotero reference manager software was used to check for duplicate articles and after the duplicates were removed, 41 articles remained. Subsequently, the titles and abstracts were screened, and 39 publications were excluded. In the final process, only two published articles were eligible for full-length review. These two articles met the inclusion criteria, and data extraction proceeded, as shown in Table 3.

Participants and exposure

Pereira et al. [2] used 30 anonymized untransformed human skin fibroblasts belonging to the COPERNIC PROJECT collection and separated them into two data sets, namely a training (n=14; 10 radiosensitive and four radioresistant) and a validation set (n=16; 11 radiosensitive and five radioresistant). Furthermore, skin biopsies were obtained from non-irradiated locations following local anesthesia using a standardized dermatologic punch. Healthy individuals lacking a cancer history or patients without adverse effects (grade 0) provided the radioresistant cells. A total of 3 hyper-radiosensitive fibroblasts were obtained from individuals with well-known genetic syndromes (Nijmegen’s breakage syndrome [NBS], Hutchinson Gilford progeria syndrome [HGPS], and ataxia-telangiectasia [A-T]) employed as negative controls. Since Pereira et al. used anonymized human skin fibroblasts, the details on the characteristics of patients were unavailable.

Deneuve et al. [5] used two populations in their study, consisting of a training and a validation cohort. The training cohort included 150 patients with a median

![Figure 1. PRISMA chart for the present systematic review](image-url)
PEG-DOX-MSN-CuS for Treating Hepatocellular Carcinoma

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Table 3. Characteristics of the studies included in this systematic review

<table>
<thead>
<tr>
<th>Study, Year, Country</th>
<th>Participants</th>
<th>Exposure and Intervention</th>
<th>Outcome</th>
<th>Main Result</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pereira et al., 2018, France [2]</td>
<td>Thirty anonymized untransformed human skin fibroblasts derived from overreactions among RT patients belonging to the COPERNIC Project collection and healthy individuals with no cancer history or cancer patients with no reaction served as the radiosensitive cells. In addition, three hyper-radiosensitive fibroblasts also derived from patients suffering from well-known genetic syndromes (Nijmegen’s breakage syndrome [NBS], Hutchinson-Gilford progeria syndrome [HGPS], and ataxia-telangiectasia [A-T]) were considered. The human skin fibroblasts were separated into two data sets: training (10 radiosensitive and four radioresistant) and validation (11 radiosensitive and five radioresistant).</td>
<td>Skin fibroblasts were exposed to 2 Gy irradiation and incubated for 10 minutes and 1 hour after irradiation. Immuno-fluorescence foci assay was used in the pATM analysis. In addition, the ELISA technique was used to quantify the pATM in irradiated and non-irradiated cells.</td>
<td>Individual radiosensitivity was categorized as an all-or-nothing reaction. RT patients who experienced NTT with a CTCAE grade greater than one were considered radiosensitive. All others (grades 0 and 1) were considered radioresistant.</td>
<td>The median of the maximal number of nuclear pATM foci in radiosensitive cell lines was significantly higher than in radiosensitive cells. The pATM ELISA data also changed significantly over the post-irradiation period and was much higher in radiosensitive cells, particularly after 10 minutes and 1 hour.</td>
</tr>
<tr>
<td>Deneuve et al., 2021, France [5]</td>
<td>The training cohort consisted of 150 RT patients [53 head and neck cancer patients, 63 prostate cancer patients, 24 breast cancer patients, five rectum cancer patients, and five other cancer patients (brain, lung, lymph node, and esophagus)], with or without concomitant chemotherapy. The validation cohort consisted of 36 non-metastatic HNSCC patients who received post-operative radiation using IMRT, VMAT, or tomotherapy, with or without concurrent chemotherapy.</td>
<td>No irradiation step was performed.</td>
<td>Patients were separated into two groups: radiosensitive (RR) patients (grade ≤2 early side effects) and radiosensitive (RS) patients (grade ≥2 early side effects).</td>
<td>The optimal pATM threshold to predict the IRS was 57.8 ng/mL for grade ≥ 2 toxicity. Based on this calculated cutoff, 48 and 52 individuals in the training cohort were properly categorized as RR and RS, while 11 and 25 subjects were well-classified as RR and RS, respectively.</td>
</tr>
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</table>

NBS, nijmegen’s breakage syndrome; HGPS, hutchinson-gilford progeria syndrome; A-T, ataxia-telangiectasia; HNSCC, head and neck squamous cell carcinomas; IMRT, intensity-modulated radiation therapy; VMAT, volumetric modulated arc therapy; LISA, enzyme-linked immunosorbent assay; pATM, phosphorylated ataxia-telangiectasia mutated; RT, radiotherapy; NTT, normal tissue toxicity; CTCAE, common terminology criteria for adverse events; RR, radioresistant; RS, radiosensitive

The study used blood lymphocytes as the cell source in subsequent investigations [5]. Lymphocytes were used because they are a less invasive sample and reduced the time between getting a sample from a patient and finding the result. Furthermore, they are nucleated cells that express pATM and can be easily isolated from blood samples. The level of pATM was also measured in the non-irradiated lymphocytes since the total level did not change following irradiation in individuals with genetic syndromes.

Outcomes and comparison

In the work of Pereira et al. [2], the severity of NTT was evaluated by two independent radio-oncologists using the CTCAE version 4.03 scale. Early and late reactions were evaluated, and the RT patients all had grade 1 to 4 NTT as adverse events (AEs). IRS was

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assumed to be an all-or-nothing phenomenon and patients who experienced AEs with a CTCAE grade of >1 were classified as radiosensitive (RS). RT patients with grades 0 and 1 were classified as radioresistant (RR).

In Deneuve et al. [5], the RT patients were categorized with early side effects graded <2 as RR individuals and those with early side effects graded ≥2 as RS individuals. By plotting the maximum number of nuclear pATM molecules against the level of clinical radiosensitivity, Pereira et al. [2] observed statistically significant differences between radioreistant cases (median 3.99 [ranging from 2.06 to 6.75] vs. median 1.49 [ranging from 0.87 to 2.17], p=0.0008). Interestingly, the subsequent study also showed statistically significant differences between patients with and without toxicity (median 58.59, ranging from 18.95 to 114.08) vs. median 48.18 [ranging from 7.61 to 80.36], p<0.0001) [5].

DISCUSSION

A brief overview was conducted followed by a description of the source of DNA damage and the repair process involving ATM protein to provide a more comprehensive explanation of DNA damage and ATM protein. This section concluded with the findings of the two studies included in the review.

DNA damage agents and source of DNA damage

The three main classes of substances acting as DNA-damaging agents are physical DNA-damaging, chemical DNA-damaging, and biological DNA-damaging substances. Physical DNA-damaging substances, including UV and ionizing radiation, can produce reactive oxygen species (ROS), which are known to damage DNA. The electromagnetic spectrum of UV radiation ranges from 100 nm to 400 nm, and it is classified into UV-A (400,320 nm), UV-B (320,290 nm), and UV-C wavelengths (290,100 nm). The ozone layer in the atmosphere often blocked UV-C light, while UV-A and UV-B radiation caused the most serious cases of DNA damage. In addition, DNA damage is caused by heat or high temperatures [8]. Different heavy metals that are considered chemical agents are responsible for DNA damage by producing DSB. Some cross-linking compounds, such as mitomycin C and cisplatin, as well as alkylating agents, also have DNA-damaging capabilities. Secondary metabolites of plants, such as nicotine, the primary alkaloid found in tobacco can promote tumorigenesis in numerous human epithelium and non-epithelial cells by producing DNA damage.

Sources of DNA damage can be roughly divided into two main groups, namely endogenous and exogenous species. Endogenous species are ROS or other agents generated by normal metabolic byproducts, while exogenous species are external agents such as radiation and solar UV radiation.

DNA damage, DNA damage repair, and ATM

The types of DNA damage induced by ionizing radiation are explained to limit the explanation to the possible types in the normal tissue of RT patients. Hydroxyl radicals generated by water radiolysis contributed 60%-70% of the cellular DNA damage induced by ionizing radiation. The damage to bases has been widely investigated in vitro by irradiating free bases, nucleosides, oligonucleotides, or DNA in an aqueous solution under aerobic or anaerobic conditions. DNA base damage was due to the oxidation of specific bases, and 8-oxo-7,8-dihydro-2′-deoxyguanine is one of the most prevalent and easily measurable products of this type of oxidation (8-oxo-dG). Hydrolytic damage to a DNA base involves the deamination, depurination, depyrimidination of individual bases, or their total removal. The addition of alkyl groups to specific bases produces alkylation products such as O2-alkylthymine, O4-alkylthymine, O6-methylguanine, and O6-ethylguanine, which may induce DNA mutations.

Another type of DNA damage induced by radiation exposure occurs when DNA strand breaks, including single-strand break (SSB) and DSB. SSB is caused by damage to the deoxyribose component of DNA and the radiation products most important are DSB, which has different degrees of complexity and is difficult to repair (Figure 2) [8,9].

There are two major DSB repair mechanisms, namely homologous recombination (HR) and NHEJ, which are considered error-free and error-prone, respectively. HR is the predominant pathway for DSB repair in yeast. In
Berthel et al. [7] presented two major hypotheses, where ATM is mainly situated in the cytoplasm as dimers formed by two autophosphorylated (pATM) monomers at serine 1981. In addition, ionizing radiation triggers the monomerization of the cytoplasmic ATM dimers in a dose-dependent manner. ATM monomers, which are active forms diffuse in the nucleus more easily than dimers and phosphorylate H2AX, triggering the NHEJ pathway. Therefore, radiation exposure induces the translocation of ATM from the cytoplasm to the nucleus called RIANS, which is an abbreviation for radiation-induced ATM nucleoshuttling.

The concept of RIANS model was used as a solid basis for IRS prediction by radiobiologists in France. Based on this concept, any delay of RIANS could lead to radiosensitivity or genomic instability, as validated by their COPERNIC Project [3]. Furthermore, a total of three groups of radiosensitivity can be defined in the population. The first is the radioresistant group, which is characterized by fast nucleoshuttling of ATM, allowing individuals to experience fast and complete DSB recognition and repair. The second is the moderate radiosensitivity group with delayed ATM nucleoshuttling, leading to incomplete DSB recognition and repair. The third is the group with hyper-radiosensitivity due to gross DSB recognition and repair defects induced by ATM or LIG4 mutations (Figures 3a-c) [3,7,14].

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pATM ELISA for IRS assay
ELISA method for quantification of pATM molecules was initially used by Pereira et al. [2] (Table 3). The study emphasized that ATM nucleoshuttling was a good predictor of human radiosensitivity. pATM

mammalian cells, NHEJ is used in a similar circumstance and is considered the major DSB repair pathway in humans [2,11]. The initiation of a cellular response, including the DSB repair process, depends on the presence of a functional ATM protein.

The study showed that ATM (mutated in A-T) protein originated from identifying people with the inherited condition A-T syndrome [12]. These individuals were also extremely sensitive to ionizing radiation, which became evident when frequently treated with RT for cancer. In addition, when A-T patient cells were grown in tissue culture, chromosomal instability and extreme sensitivity to DSB-inducing agents, such as ionizing radiation and radiomimetic chemical agents, were observed. In ATM-deficient cells, the responses were attenuated or delayed but not absent. This suggests that ATM kinase is required for the initial, and rapid response to damage, even though other enzymes may initiate similar pathways.

The key regulators of DNA damage repair are members of the phosphatidylinositol 3-kinase-like protein kinase family. This family contains the enzymes ATM, A-T, RAD3-related, and DNA-dependent protein kinase catalytic subunit. Following the recruitment to DSB sites by the MRN complex, ATM proteins are activated by autophosphorylation. The activation of ATM results in the phosphorylation of a large number of downstream effectors, including checkpoint kinase 2 and histone H2AX (γ-H2AX). CHK2 activation may cause a checkpoint arrest in G2/M to allow DNA repair or trigger apoptosis. Due to the activation by ATM, γ-H2AX binds to DNA break flanking areas of up to a hundred kilobases and indicates lesions as an early sensor of DSB through the NHEJ pathway (Figure 2) [10,13].
imunofluorescence assay requires a process of cellular amplification and one to three weeks to produce adequate quantities of cells.

pATM ELISA can be integrated into an automatic high-throughput screening. Recently, Deneuve et al. [5] developed the quantification of total pATM using ELISA on another cell type of RT patients (with peripheral blood lymphocytes) for the IRS assay known as the RADIODETECT® assay. The lymphocytes were used to develop a faster, clinically pragmatic, and less invasive assay. The RADIODETECT® assay consists of three steps, namely lymphocyte isolation, cell lysis, and pATM quantification using a RIPA buffer and ELISA Kit (Figure 4).

This assay can predict IRS in RT patients regardless of confounding factors such as gender and cancer type. Radiosensitivity prediction is a crucial step in personalizing radiation treatments. According to the two publications, pATM ELISA is a promising method for predicting IRS in RT patients. However, the use of concurrent chemotherapy should be considered when evaluating the risk of toxicity in RT patients. Concerning the limitations, the small proportion of patients who received concurrent chemotherapy should be validated with other investigations in larger cohorts.

CONCLUSIONS

This systematic study was conducted to provide information on the use of pATM ELISA to predict IRS and NTT in RT patients. It was reported that two publications fulfilled the inclusion criteria, where pATM ELISA assays, involving lymphocytes (called RADIODETECT®) predicted the risk of early NTT in RT patients.

DECLARATIONS

Competing interest
The authors declare no competing interest in this study

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