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The pATM Immunofluorescence assay: a high-performance radiosensitivity assay to predict post radiotherapy overreactions

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Running title:

Updated performance of the pATM radiosensitivity assay

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Conflicts of interest

GV and AC have nothing to disclose.

TB is Co-Founder, Scientific Expert in Biostatistics and Nano-informatics in CYBERNANO company (Biosignal Processing & Biostatistics).

SP and LB report the following patents: FR3040178A1 FR3040179A1 WO2017098190A1 WO2017029451A1 WO2017029449A1 licensed to NEOLYS Diagnostics. SP is Head of R&D at NEOLYS Diagnostics. JGD is co-founder and CEO of NEOLYS Diagnostics.

NF reports the following patents: FR3040178A1, FR3040179A1, FR3017624A1, FR3017625A1, WO2015121597A1, WO2017098190A1, WO2017029451A1, WO2015121596A1, WO2017029449A1. NF is co-founder and Scientific Advisor of NEOLYS DIAGNOSTICS.

Authorship

GV drafted the manuscript and helped in the acquisition of data. AC and LB performed the primary statistical analyses. TB performed the secondary analyses. NF, JGD and SP interpreted the data. All authors revised the manuscript critically and have approved the final article.

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The pATM Immunofluorescence assay: a high-performance radiosensitivity assay to predict post radiotherapy overreactions

Running title: Updated performance of the pATM radiosensitivity assay

SUMMARY

A new statistical analysis was performed on 117 patient-specific fibroblasts lines cultured from a skin biopsy in order to assess AUC, sensitivity, specificity, the positive predicted value and the negative predicted value of the pATM Immunofluorescence assay to sort overreactors. The pATM assay shows the highest predictive performance amongst the available tests.

ABSTRACT

Purpose

Identifying prior treatment, the patients who will overreact to radiotherapy (RT) would have sound positive clinical implications. By focusing on DNA double-strand breaks (DSB) recognition and repair proteins after irradiation, we recently demonstrated that the maximal number of pATM nuclear foci in the first hour (pATMmax) after ex vivo irradiation correlated with post-RT toxicity severity. We aimed to carry out additional analyses on our whole collection of fibroblast lines to refine the predictive performance of our assay.

Material and Methods

30 Immunofluorescence experiments were performed on 117 primary skin fibroblast lines irradiated at 2 Gy. The
31 toxicity response was split up into two binary classes: 0 if toxicity grade < 2 and 1 otherwise. To assess the
32 relationship between the quantity of pATMmax foci and toxicity grade, we applied a correlation then a supervised
33 classification analysis. Training datasets from 13 radiosensitive patients randomly drawn with a random under-
34 sampling technique were constituted. ROC analyses were performed with a Monte-Carlo method to estimate the
35 optimal threshold and discriminate the responses for each data set. The discrimination cut-off was estimated as
36 the maximum value of the 10^4 thresholds computed from each training subset.

37 Results

38 As expected, we confirmed a quasi linear dependence between toxicity and pATMmax (Fig 1A & 1B; Pearson
39 correlation coefficient: -0.85; $p < 2.2e^{-16}$). When taken as a binary predictive assay with the optimal cut-off value
40 of 34.5 pATM foci/cell, our assay showed an outstanding predictive performance with the following values
41 respectively for sensitivity, specificity, NPV, PPV and AUC: 1.00, 0.92, 1.00, 0.99 and 0.987.

42 Conclusion

43 The results of these experiments allowed us to identify pATMmax as a high performance predictive parameter of
44 post-radiotherapy OR. Additional studies are in progress to confirm that this radiosensitivity assay reaches the
45 same performances in any condition to adapt the clinical practice.

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49 **MANUSCRIPT**

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53 **Introduction**

54 Up to 15% of the patients treated with radiotherapy (RT) experience toxicity that can lead to serious sequelae and
55 additional societal costs (1-3). In particular, their cumulative incidence at least 30 years after the diagnosis of
56 cancer reaches 73.4% in pediatric oncology (4). Identifying these over reactors (OR) prior RT would therefore
57 have sound positive clinical implications.

58 The severity of toxicity depends on several factors dominated by individual radiosensitivity (iRS) (5). iRS could be
59 defined as the innate capacity of a given individual to show a particular toxicity profile to radiation - corresponding
60 to the loss of proliferative capacity observed in vitro.

61 Since 2003, we have collected primary skin fibroblasts from patients with DNA repair deficiencies, from apparently
62 healthy patients or from irradiated patients with various severity grades and free intervals after RT in more than 30
63 French or Belgian hospitals. According to Baumann et al., OR are unselected patients who experienced severe
64 side effects defined as follows, after the same standardized physical radiation dose, without evidence of
65 treatment- or patient-related cofactors that can affect radiation tolerance (6) :

- 66 • grade ≥ 2 occurring the first two weeks of RT where these side effects are unexpected or
67 • grade ≥ 3 lasting more than 4 weeks after the end of RT or
68 • grade ≥ 3 occurring or persisting more than 90 days after the end of RT (7).

69 We measured the RT-induced distribution of candidate DNA double-strand breaks (DSB) recognition and repair
70 proteins with an immunofluorescence assay (8). We thus recently proposed a general classification of Human iRS
71 based on the nucleoshuttling of ATM kinase (9). However, we described a continuous distribution of the maximal
72 number of pATM nuclear foci in the first hour (pATMmax) after ex vivo irradiation according to the 6 possible
73 severity grades taken individually – that is not suitable to support clinical decision making (9). During ESTRO36,
74 we reported a preliminary ROC curve analysis according to a binarized toxicity grade classification. With the
75 optimal cut-off value of 35 foci, pATMmax assay showed promising predictive performances, with an area under
76 the curve (AUC) of 0.97, a predictive positive value (PPV) of 99%, a specificity of 92% and a sensitivity of 100%
77 (10).

78 Our objective in the present letter was to refine the performance of our iRS assay on the whole cohort applying a
79 new analysis with a robust supervised classification.

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83 **Material and Methods**

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85 Material description

86 This study was conducted on the 117 primary skin-untransformed fibroblast lines we previously reported including
87 12 radioresistant, 4 ATM^{-/-}, and 1 LIG4^{-/-} gifted cell lines and 100 lines from OR patients (9). Among the
88 irradiated patients in our cohort, the median time from treatment to biopsy was 13.3 months (1-273). Two
89 independent expert radiation oncologists graded the toxicity severity according to the Common Terminology
90 Criteria for Adverse Events (CTCAE) version 4.03. The toxicity response was split up into two binary classes: 0 if
91 toxicity grade < 2 (“biologically radioresistant”) and 1 otherwise (“biologically radiosensitive”)

92 The regional ethical committee approved the COPERNIC collection. Cell lines were declared under the numbers
93 DC2008-585 and DC2011-1437 to the French Ministry of Research. The database was protected under the
94 reference as IDDN.FR.001.510017.000.D.P.2014.000.10300. All the patients were informed and gave signed
95 consent according to the ethics recommendations.

96 The biological methods have been extensively described (8, 11).

97 Statistical analysis

98 **Assessment of quality.** To assess the relationship between the quantity of pATMmax foci – defining iRS - and
99 binarized toxicity grade, we applied a Pearson correlation test. The intra-grade variance is defined as the variance
100 of pATMmax foci in each CTCAE group. A Student t-test and a Fisher test were carried out to examine the
101 relevance of the affine model parameters and the adequacy between the model response and the experimental
102 data respectively. In each test, the associated p-value indicates the compatibility of data with the null hypothesis.

103 **Supervised classification.** Our initial dataset was expected unbalanced between “radiosensitive” and
104 “radioresistant” patients. To correct it, a random under-sampling technique was implemented, in which 13
105 radiosensitive patients were randomly drawn from the 104 available subjects (12). As a consequence, each
106 training dataset was first composed of 26 patients (13 radiosensitive and 13 radioresistant patients). The thirteen

107 radioresistant subjects were always the same in this study. ROC analyses were performed with a Monte-Carlo
108 method to estimate the optimal threshold and discriminate the responses for each data set. Finally, the
109 discrimination cut-off was estimated as the median value of the 10^4 thresholds computed from each training
110 subset. The classification performances were characterized on the 117 patients by five parameters: AUC,
111 sensitivity index, specificity index, the PPV and the negative predicted value (NPV). The analyses were carried
112 out on R.

113

114 **Results**

115 Two independent expert radiation oncologists defined the severity grade in blind with total agreement.

116 Bivariate Analysis

117 As expected, we clearly emphasized a quasi-negative linear dependence between toxicity and pATMmax (Fig 1A;
118 Pearson correlation coefficient: -0.85). The t-test and F-test confirmed the strong correlation between those two
119 quantities reflecting the degradation of pATM nucleoshuttling with worsening of toxicity (p-values $< 2.2e^{-16}$ for the
120 two tests).

121 Supervised classification analysis

122 We reclassified 104 radiosensitive patients and 13 radioresistant subjects. After the Monte-Carlo session, the final
123 classification cut-off was estimated at 34.5 pATMmax foci/cell nucleus. From the ROC curve, we obtained the
124 following values respectively for the sensitivity, specificity, NPV, PPV and AUC: 1.00, 0.92, 1.00, 0.99 and 0.987
125 (Fig 1B). We estimated the coefficients of variation of each quality criterion in the same order: 0, 0.019448,
126 0.023315, 0 and 0.0076695.

127

128 **Discussion**

129 To our knowledge, our pATMmax predictive assay performed on skin fibroblasts show the highest performance
130 reported so far – pointing ATM nucleoshuttling as a very good predictor for Human iRS (13-15). This quantitative
131 correlation was found to be independent of tumor location, cell type and of early or late occurrence of the toxicity
132 (9). Unlike other assays reporting molecular or cellular endpoints like polymorphisms or apoptosis, our assay
133 provides: 1) a reliable prediction of radiosensitivity by considering CTCAE grades separately – and in a binarized
134 way (9); 2) a relevant biological interpretation of the linear-quadratic model (16-18); 3) a relevant mechanistic

135 model to explain major IRS in genetic diseases associated with mutations of cytoplasm proteins interacting with
136 pATM nucleoshuttling (11, 19, 20). Patients are currently enrolled prior RT in confirmatory prospective studies in
137 pediatrics (NCT02827552) and sarcomas/GI malignancies (NCT02797405).

138 We are aware that the mandatory cellular amplification step after skin sampling requires several weeks that could
139 appear not suitable for clinical use. However, we are now developing a fast ELISA-assay targeting pATM directly
140 from the original skin sample and with still very interesting performances (average AUC of more than 0.8) and a
141 deadline of a few days for editing the results (14).

142

143 Conclusion

144 When taken as a binary predictive assay, the pATMmax predictive assay appears as one of the most powerful
145 available. Prospective studies are in progress to confirm its performance.

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149 **FIGURE CAPTION**

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151 A. Correlation analysis between the mean number of pATM foci/cell nucleus and the individual CTCAE grades.
152 Each cross denotes a radioresistant patient and each circle a radiosensitive subject. The red line represents the
153 regression providing the best fit to experimental data. B. ROC curve computed from the whole reclassified data
154 set, AUC=0.987 (CV=0.0076695)

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