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Use a survival model to correlate single-nucleotide polymorphisms of DNA repair genes with radiation dose response in patients with non-small cell lung cancer

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Abstract

Purpose—This study utilizes a survival model and clinical data with various radiation doses from prospective trials to determine radiation dose response parameters, such as radiosensitivity, and identify single-nucleotide-polymorphism (SNP) biomarkers that can potentially predict the dose response and guide personalized radiotherapy.

Methods—The study included 92 consecutive stage-III NSCLC patients with doses varied from 60 to 91 Gy. Logistic regression analysis of survival varying with SNP genotype and radiation dose was used to screen candidates for dose response analysis. The dose response parameter, represented by D_{50} , was derived by fitting survival data into a model that takes into account both tumor control and treatment mortality. A candidate would be considered as a predictor if the 90% confident intervals (90% CIs) of D_{50} for the 2 groups stratified by the SNP genotype were separated.

Results—One SNP-signature (combining ERCC2:rs238406 and ERCC1:rs11615) was found to predict dose response. D_{50} values are 63.7 (90% CI: 53.5–66.3) Gy and 76.1 (90% CI: 71.3, 84.6) Gy for the 2 groups stratified by the genotypes. Using this biomarker-based model, a personalized dose prescription may be generated to improve 2-year survival from ~50% to 85% and ~3% to 73% for hypothetical sensitive and resistant patients, respectively.

Conclusions—We have developed a survival model that may be used to identify genomic markers, such as ERCC1/2 SNPs, to predict radiation dose response and potentially guide personalized radiotherapy.

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Keywords

personalized radiotherapy; single-nucleotide-polymorphisms (SNPs); Biomarker; ERCC1; ERCC2; dose survival model; radiosensitivity

Introduction

Lung cancer is the leading cause of cancer death [1]. Although single-institution studies and secondary analysis of RTOG patients indicated that higher dose radiotherapy (RT) may increase local control and survival for patients with non-small cell lung cancer (NSCLC) [2–4], the RTOG 0617 study showed a contradictory result [5]. The overall survival (OS) in the high-dose arm (74-Gy) was poorer than that of the low-dose arm (60-Gy). These controversial results suggest a heterogeneous dose response of the patients, and the need of biomarkers to identify a patient's sensitivity of dose response to RT for personalized medicine. For sensitive patients, 60-Gy may be sufficient to control the tumor, and increasing dose will only harm the patients by increasing treatment toxicity. On the other hand, for resistant patients, even 74-Gy may not be sufficient to control the tumor.

The radiation dose response, or radiosensitivity of a patient, depends on many factors, such as the DNA repair capability of tumor cells after radiation damage [6]. DNA repair genes play an important role in the processes of repairing these radiation damages through different pathways, and thus may correlate with a patient's radiosensitivity. Recently, studies have shown that single nucleotide polymorphisms (SNPs) of DNA repair genes correlated with treatment response and OS for NSCLC patients with chemo-radiation treatment [7–9]. However, reports are often inconsistent [8]. One speculation of these inconsistencies is that OS or treatment outcome is the combined result of both tumor and normal tissue responses. A patient's intrinsic DNA repair capability may affect both tumor and normal cells, so that its relation to treatment outcome is complicated by the heterogeneous chemo-radiation doses. Sensitive patients may have better outcome when the dose of a chemo-radiation regimen is low, while resistant patients may have better outcome when the dose is relatively high.

Correlation of radiosensitivity with biomarkers has not been successfully explored previously in patients, possibly because of its extraordinary challenges. Radiosensitivity is not an outcome parameter like OS. It cannot be measured for each individual patient. The average radiosensitivity of a group of patients, which is represented by a parameter D_{50} , may be determined by fitting a sigmoid-shaped dose-response curve. However, a relatively large amount of patient data with various radiation doses, which are usually not clinically available, are required to determine the dose response curve. In addition, the tumor response is usually estimated by local tumor control, which may not be assessed objectively [4], especially for patients who died earlier from non-local tumor causes. Survival or death is objective, but confounded by many other factors, including (but not limited to) patient comorbidities, treatment toxicities and tumor metastasis. With a stage III NSCLC patient data set of various RT doses in prospective protocols, this study aims to evaluate the feasibility of utilizing OS as the surrogate endpoint for tumor response to derive the D_{50} .

and determine its association with a SNP marker by comparing the D_{50} values for the 2 groups of patients stratified by the marker. Specifically, we will 1) develop an OS model, which takes into account of not only the tumor control, but also other factors such as the patient comorbidities, treatment toxicities, and tumor metastasis, for deriving the D_{50} values; 2) identify SNP markers that are associated with the radiation dose response; and 3) presents a methodology of utilizing the SNP markers for personalized dose prescription to improve survival.

Materials and Methods

Study population

All stage-III NSCLC patients who enrolled in our institutional prospective protocols, and had blood samples when the SNP test was carried out, were eligible for the study. A total of 92 patients were eligible. The patients were mainly from 3 protocols, which have been all approved by IRB for biomarker correlation studies. The first is a dose escalation protocol using a PET-guided adaptive RT technique, with prescription dose limited by an iso-toxicity criterion, similar to that described in the RTOG 1106 study. The dose varied from 74–91 Gy in this protocol. The second is also a dose escalation study using a similar iso-toxicity criterion, but without advanced adaptive RT technique. The radiation dose was typically in the range of 66–80 Gy. The third is an imaging and biomarker protocol with standard RT. The radiation dose was typically 60–74 Gy (the high dose patients were treated before RTOG 0617 was completed). Dose tolerance criteria of organs-at-risk (OARs) similar to the iso-toxicity criterion were used for the patients in the third protocol. Those patients who did not finish treatments were also included in the study. Minimum follow-up was 2 years. Table 1 lists the basic properties of the patients.

SNP/SNP-signature candidates

Blood samples were collected for each patient before RT. DNA was isolated from buffy coat, and a Sequenom MassArray System (Sequenom, Inc, San Diego, CA) in the Biomedical Research Core Facility at University of Michigan was used for SNP genotyping. A total of 24 functional SNPs in 11 DNA repair genes were tested. These include 7 ATM (rs1800057, rs189037, rs373759, rs609261, rs664677, rs664143 and rs4988044), 3 ERCC1 (rs11615, rs3212948 and rs3212961), 1 ERCC2 (rs238406), 2 ERCC5 (rs17655 and rs1047768), 1 XRCC1 (rs25489), 2 XRCC2 (rs3218536 and rs6464268), 3 XRCC4 (rs1478486, rs2075685 and rs9293329), 2 XPC (rs2228000 and rs2228001), 1 MGMT (rs12917), 1 NBN (rs1805794) and 1 TP53 (rs1625895) SNPs

The following 3 steps were used to screen SNP/SNP-signature candidates for dose response analysis. 1) All the SNPs were tested for the difference of OS rates of any 2 groups stratified by the SNP genotypes, and those with large differences ($>15\%$ and $p<0.3$) were advanced to the next step. 2) SNP-signatures were generated by combining any two of these SNPs using the “or” or “and” operator. However, usually only the “or” was used to increase the patient number for the group with less patients (usually the high-OS-rate group) so that patient numbers were more balanced for the 2 groups stratified by the SNP signature. SNPs and SNP-signatures were tested for the statistic correlation with the OS using Fisher’s Exact

Test. Those significantly correlated with OS were advanced to the next step. (3) A conventional logistic model of OS with 2 variables, including the biomarker as a category variable, and the radiation dose as an explanatory variable, was fitted to the data to test if the biomarker and radiation dose were both statistically significant predictors of the OS. Those biomarkers that were significant were considered as candidates for the final OS model based dose response analysis.

OS model for dose response analysis

OS depends not only on local tumor control, but also many other factors. We divided these factors into 3 categories: (1) tumor response to RT; (2) normal tissue response to RT; and (3) all other factors not relating to RT. The factor for tumor response to a RT regimen can be modeled as tumor control probability (TCP) using a dose-dependent sigmoid-curve function. The factor for normal tissue response to the RT can be considered as treatment mortality and modeled as normal tissue complication probability (NTCP) using a similar sigmoid function. The other factors not relating to the RT can be patient factors, such as other diseases, aging, presence of micro-metastasis during treatment, susceptibility to metastasis, and so on, or other treatment factors such as chemotherapy. These non-RT related factors may be represented as a combined prognostic factor, $P(B, t)$, which depends on many variables as well as biomarker status B and the follow-up time t . For simplicity, those variables were not specifically expressed in the formalism because the model was used for biomarker related dose response analysis. Thus, the OS rate for a group of patients is the multiplication of the contributions of the 3 sources, and can be expressed as:

$$OS(D, B, t) = P(B, t) \cdot \frac{1}{1 + [D_{50}(B)/D]^{\lambda(B)}} \cdot \left[1 - \frac{1}{1 + [D^{(N)}_{50}(B)/D^{(N)}]^{\lambda^{(N)}(B)}} \right] \quad (1)$$

where the 3 terms at the right correspond to contributions of combined prognosis, TCP and NTCP, respectively; B is the biomarker status; $D_{50}(B)$ and $D^{(N)}_{50}(B)$ represent the radiosensitivity of the tumor and the normal structures, respectively; $\lambda(B)$ and $\lambda^{(N)}(B)$ reflect the degree of their homogeneity in radiosensitivity and dictate the slope of the sigmoid curves; $D^{(N)}$ is the equivalent reference dose to the combined normal structures, including lung, heart, esophagus, and the immune system.

In this study, we used mean lung dose (MLD) as the equivalent reference dose for the combined normal structures to simplify the model, because MLD is significantly correlated to the esophagus, heart and other organ doses [10]. Considering iso-toxicity was generally applied for this group of patients, we assumed that $D^{(N)}$ was a constant. For convenience, 2-year OS was used in this study. Thus, Eq. (1) is simplified as:

$$OS(D, B) = \frac{k(B)}{1 + [D_{50}(B)/D]^{\lambda(B)}} \quad (2)$$

where $k(B)$ represents the multiplication of the combined prognosis and normal tissue response. It should be pointed out that tumor volume has been reported correlating to survival [11–12] and may indirectly relate to the dose. The effect of tumor volume to

survival could be included in Eq. (1) for all 3 terms: (1) it may have a non-dose related prognosis included in $P(B,t)$; (2) it may impact $D_{50}(B)$; (3) it impacts $D^{(N)}$ because large tumor volume usually results in high $D^{(N)}$. The iso-toxicity has normalized the impact of the tumor volume to $D^{(N)}$ in our data set. Again, because the correlation of tumor volume to survival was not the focus, it was not specifically expressed as a variable in $P(B,t)$ and $D_{50}(B)$.

Eq. (2) presents the OS model to determine $D_{50}(B)$ for a group of patients. The patient data can be displayed in 2 different ways to fit the model. The first is to display calculated OS rates by dividing patients into several dose groups and calculating OS rate for each dose group. We used this approach to evaluate the goodness of fit (R^2) of the model with patient data. However, due to the nature of retrospective study and limited number of patients, the division of the dose groups is often subjective. Thus, the approach is not statistically rigorous for deriving the fitting parameters. The second is to display OS rate as binary data (either 1 or 0) for each patient. This is a standard statistical approach and is also called binary approach. However, due to the nature of binary data, we are not able to illustrate how well the model fit with the data. We used this approach to determine the 3 parameters, $D_{50}(B)$, $\lambda(B)$, and $k(B)$, as well as their corresponding confidence intervals (CIs). An in-house computer program that utilizes the fitting functionality in the R software (a shared online statistical software platform) [13] was used to perform the fitting analyses. The model was first applied in the combined group (or mixed group) including all 92 patients, and then for the 2 groups stratified by each biomarker candidate. We considered that a biomarker candidate is associated with radiosensitivity if the 90% CIs of the D_{50} for the 2 groups stratified by the biomarker are not overlapped. The group with the lower D_{50} value was defined as the radiosensitive group.

OS model for personalized RT

The OS model has the potential to directly guide personalized RT if the radiosensitivity parameters can be determined. However, the simplified model of Eq. (2) does not include $D^{(N)}_{50}(B)$, $\lambda^{(N)}(B)$ and $D^{(N)}$, the normal tissue related parameters due to the iso-toxicity prescription. We derive them by relating them to $D_{50}(B)$, $\lambda(B)$ and D , the tumor related parameters. Because SNPs reflect genetic characteristics of an individual, both tumor and normal tissue cells would undergo the same degree of DNA repair and share similar sensitivity. Thus, we have $m = D_{50}(B)/D^{(N)}_{50}(B)$ and $n = \lambda(B)/\lambda^{(N)}(B)$ being constants not relating to “B”. In addition, the biomarker predicting radiosensitivity may not correlate to combined prognosis. Thus, $P(B,t) = P(t=2\text{-years}) = P_0$. We further define $S_F = D/D^{(N)}$ as an OAR sparing factor to reflect a RT plan’s capability of sparing the normal tissue. S_F would depend on the tumor volume, tumor location and RT technique. Thus, the OS model of Eq. (1) becomes

$$OS(D, B, S_F) = P_0 \bullet \frac{1}{1 + [D_{50}(B)/D]^{\lambda(B)}} \bullet \left[1 - \frac{1}{1 + [S_F \bullet D_{50}(B)/m/D]^{\lambda(B)/n}} \right] \quad (3)$$

And we have

$$k(B) = P_0 \bullet \left[1 - \frac{1}{1 + [D_{50}(B)/m/D^{(N)}]^{\lambda(B)/n}} \right] \quad (4)$$

Three sets of $D_{50}(B)$, $\lambda(B)$ and $k(B)$ values were obtained by fitting Eq. (2) for 3 different B statuses (mixed group, and 2 groups stratified by the biomarker). Plugging these values into Eq. (4), and assuming mean lung dose (MLD) = $D^{(N)}$, we had 3 equations with 3 unknowns, m , n and P_0 . The 3 unknowns were estimated by solving the 3 equations. Thus, Eq. (3) can predict OS for any patient with a given D , B , and S_F .

Results

Four SNPs were considered as candidates in the first step due to their relatively large difference of OS rates between the 2 groups stratified by the candidates, as shown in Table 2. Among them, ERCC1:rs3212948 almost exactly correlated with ERCC1:rs11615. All 12 patients with the GG-type in rs3212948 were CC-type in rs11615, which has 13 patients. Therefore, the rs3212948 can be represented by the rs11615 and was not further analyzed. One SNP (ERCC2_rs238406), and 2 SNP-signatures (rs238406/rs11615 and rs238406/rs9293329) were significantly correlated with OS ($p = 0.005$, 0.006 , and 0.01 , respectively), as also shown in Table 2. The logistic regression analysis showed that the SNP (rs238406) and a SNP-signature (rs238406/rs11615) were dose-dependent significant predictor for the OS ($p = 0.03$ and 0.03 respectively), and were considered as the final candidates for dose response analysis.

Fig. 1 shows the results of model-based dose response analyses for the two candidates. We first demonstrated that the model fits the clinical data very well for all situations using the dose-group approach, with $R^2 = 0.98$ – 0.999 , as shown in Figs. 1A and 1B. Using the binary approach, we derived the 3 parameters, $D_{50}(B)$, $\lambda(B)$, and $k(B)$, and their corresponding 90% CIs for the mixed group, as well as the two groups stratified by each biomarker candidate. The fitting curves are shown in Figs. 1C and 1D, and the parameters are listed in Table 3. We note that for the 2 groups stratified by the SNP-signature, their D_{50} values (and 90% CIs) are 63.7 (53.5–66.3) Gy and 76.1 (71.3, 84.6) Gy, respectively. The 90% CIs are not overlapped, suggesting that the SNP-signature is associated with radiosensitivity according to our criterion. On the other hand, for the 2 groups stratified by the single SNP candidate, the D_{50} values (and 90% CIs) are 64.3 (55.4–77.9) Gy and 82.7 (70.5, 95.1) Gy, respectively. Although the D_{50} values are very different, the 90% CIs are overlapped, suggesting our data is not sufficient to support that the SNP rs238406 is a biomarker that associates with radiosensitivity.

Using the data in Table 3, we estimated that $P_0 \approx 85\%$, $m \approx 3.64$, and $n \approx 0.95$. Thus, the model represented by Eq. (3) could be used to predict the 2-year OS and guide personalized RT for an individual patient. Fig. 2 shows examples of how to use the model to guide personalized RT by plotting OS varying with dose for different biomarker statuses, and for 2 typical hypothetical patients with different S_F values ($S_F = 4.0$ and 4.7). The optimal prescription dose and corresponding OS differ remarkably for different SNP-signature statuses and S_F values. Two-year OS reaches 85% for the radiosensitivity, and 73% for

radioresistant status for a patient with $S_F = 4.7$ at optimal prescription dose. However, it is ~50% and ~3%, correspondingly, for use of a uniform prescription of ~64 Gy.

Discussion

We have developed an OS model that can well fit to the clinical data and be used to derive the radiosensitivity parameters from a group of patients. Based on this model, we have also developed a methodology that can determine whether a biomarker candidate is associated with radiosensitivity. Using this methodology, we have identified a SNP-signature, a combination of ERCC2: rs238406 and ERCC1: rs11615, to be such a biomarker, because the D_{50} values and their 90% CIs are widely separated for the 2 groups of patients stratified by this biomarker. Logistic model based statistical analysis showed that this SNP-signature and the radiation dose are significant factors associated with OS, consisting with the result that the SNP-signature is associated with radiosensitivity. Finally, we have illustrated that a combination of this model and the biomarker data may be used to predict a patient's survival and potentially guide personalized RT, as shown in Fig. 2. It should be pointed out that iso-toxicity is a key in deriving the sigmoid-shaped dose response model for the data analysis. We noted that MLDs were relatively uniform in these patients (16.8, 17.0, 16.0, 16.2, and 16.1 Gy, respectively, for the 5 dose groups), although with a large dose variation (60–91 Gy).

The ERCC1 and ERCC2 genes are well known for repairing ultraviolet-radiation induced DNA damage through the nucleotide excision repair (NER) pathway [14–16]. It has also been reported that they were correlated with platinum-based chemotherapy for treatment response, survival [7–8, 17] and treatment toxicities [18–20] due to their role in the NER pathway. Therefore, they are more likely to modulate chemo or ultraviolet-radiation response than RT response. However, these genes may be involved in transcription, and thus may contribute to repair of other types of damage, such as double strand breaks by ionizing radiation [15]. In addition, ionizing radiation also induces oxidative damage similar to ultraviolet radiation and chemotherapy. Therefore, it is understandable that these genes may be associated with radiosensitivity. Studies have shown that the ERCC1 gene was involved in double strand breaks [21] and correlated with radiosensitivity in glioma cell lines [22], and the SNPs of ERCC2 correlated with repair of x-ray induced DNA damages, such as chromatid aberrations [15] and chromosome dicentrics [16]. The ERCC2:rs238406 and ERCC1:rs11615, along with their highly associated tag-SNP [23], have also been reported to correlate with repair of Benzo[a]pyrene-diol-epoxide (BPDE)-induced DNA adducts in cultured primary lymphocytes [24–25]. It is also interesting to note that the genotype frequency of both rs238406 and rs11615 highly depends on race [26]. The GG type in rs238406, and CC type in rs11615, which correspond to radioresistance, have high frequency in Africans (>90%), intermediate in Asians (20–60%), and low in Europeans (7–20%) [26]. The majority of patients in this study were Caucasians.

The parameter k , which inversely relates to treatment mortality, is 0.61 for sensitive patients, and 0.83 for resistant patients stratified by the SNP signature. This suggests that patients with radiosensitive tumors may have radiosensitive normal tissue. We noted that at MLD ~17 Gy, treatment mortality reached >24% for radiosensitive patients. However, we did not

observe such a large number of grade-5 toxicities of specific anatomic organs. One speculation is that the NTCP term in the model (Eq. 1) may also include radiation damage of the immune system. Recently, it has been reported that the immune system plays an important role in controlling the tumor [27–30]. Damage of the immune system may not directly cause conventional treatment mortality, but may affect tumor control and eventually patient death due to disease progression.

A major limitation of this study is the relative small patient data set. The patient number is not sufficient to separate them into a training and a validation cohort for statistical validation. Another caveat in this study is that this is a retrospective study including patients from 3 different protocols. Although all eligible Stage 3 patients were included, the enrollment criteria for the 3 protocols may be different, and different iso-toxicity criteria might be used for different protocols. In addition, the impacts of chemotherapy, tumor volume, and metastasis to the survival and the model were not systematically studied and not clearly understood. Due to the limited patient number, especially at the high radiation dose, it is possible that the correlation of SNP-signature with the dose response is false positive. Considering that 88% of patients underwent platinum-based chemotherapy, and the SNP-signature is well known for DNA repair in the NER pathway, the false positive could be a reflection of a prognostic predictor for chemotherapy. Further studies with a larger number of patients are needed to validate the SNP/SNP signature, and improve accuracy of the dose response parameters in the model.

In summary, we have developed an OS model that can fit to the clinical data well and can derive the radiosensitivity parameters from a group of patients. Based on this model, we have further developed a methodology that can identify whether a biomarker candidate, such as a SNP signature that combines ERCC2: rs238406 and ERCC1: rs11615, can predict radiosensitivity. This model, with inclusion of the biomarker, if validated, might be used for personalized RT to improve treatment outcome.

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Fig 1.

Dose-survival relationship for the 2 SNP/SNP-signature candidates and their fits with the model for 2 approaches of displaying patient data: (A) Single SNP/Approach 1; (B) SNP-signature/Approach 1; (C) Single SNP/Approach 2; (D) SNP-signature/Approach 2.

Approach 1 (Figs. A&B) displays 2-year OS in 5–6 data points by dividing patients into 5–6 dose groups; and the purpose is to illustrate how well the model fits to patient data.

Approach 2 (Figs. C&D) plots OS as binary data (1/0) for each patient; and the purpose is to derive the fitting parameters. The patient OS rate data from approach 1 are also plotted in Figs. C&D for references.

Fig. 2.

Dose-OS response curves predicted by the OS model, which varies with dose D , biomarker genotype B and OAR sparing factor S_F . (A) for a virtual patient with $S_F=4.0$; (B) for a virtual patient with $S_F=4.7$.

Table 1

Characteristics of the patients

Characteristics		Number (%)
Gender	Male	75 (82)
	Female	17 (18)
Race	Caucasian	78 (85%)
	Black	4 (4%)
	Others and Unknown	10 (11%)
Age	<65	43 (47)
	≥65	49 (53)
Histology	Squamous	23 (25)
	Adenocarcinoma	19 (21)
	Others	50 (54)
Smoke	Yes	88 (96)
	No	4 (4)
Chemo (carboplatin + Paclitaxel)	Yes	81 (88)
	No	11 (12)
COPD	Yes	39 (42)
	No	53 (53)
Kps: Median (range)	90 (50–100)	
GTV: Median (range)	164.7 (2.4–921) CC	

Table 2

The 4 SNP candidates advanced from Step-1 to Step-2 to form SNP-signatures, and the 2 SNP-signatures advanced from Step-2 to Step-3.

SNPs or SNP-signatures	Genotypes	Patient number (% of patient)		P value
		Distribution	Two-year OS	
ERCC2: rs238406	GG	30 (33%)	6 (20%)	0.005
	not GG (GT+TT+0)	62 (67%)	31 (50%)	
ERCC1: rs11615	CC	13 (14%)	3 (23%)	0.07
	not CC (CT+TT+0)	79 (86%)	34 (43%)	
ERCC1: rs3212948	GG	12 (13%)	3 (25%)	0.20
	not GG (CG+CC+0)	80 (87%)	34 (43%)	
XRCC4 rs9293329	AG	11 (12%)	3 (27%)	0.28
	not AG (AA+GG+0)	81 (88%)	34 (42%)	
Signature1:rs238406/rs11615	GG in rs238406 or CC in rs11615	38 (41%)	9 (24%)	0.006
	The others	54 (59%)	28 (52%)	
Signature2:rs238406/rs9293329	GG in rs238406 or AG in rs9293329	36 (39%)	9 (25%)	0.01
	The others	56 (61%)	28 (50%)	

Table 3

Model Parameters and Goodness of Fit of the SNP and SNP-signature candidates.

Biomarker Name	Biomarker Status	D_{50} (90% CI) (Gy)	λ (90% CI)	k (90% CI)	R^2
Single SNP rs238406	Sensitive	64.3 (55.4, 77.9)	22.4 (4.6, ND)	0.64 (0.49, ND)	0.12
	Resistant	82.7 (70.5, 95.1)	10.9 (4.3, ND)	1.0 (0.43, ND)	0.37
SNP signature	Sensitive	63.7 (53.5, 66.3)	50 (6.8, ND)	0.61 (0.48, ND)	0.18
	Resistant	76.1 (71.3, 84.6)	19.5 (8.0, ND)	0.83 (0.59, ND)	0.49
Mixed group		67.2 (62.2, 80.8)	13.8 (4.8, 45)	0.67 (0.51, ND)	0.17

ND = Not determined due to limited data points