Published in final edited form as:

Radiother Oncol. 2010 October ; 97(1): 9–14. doi:10.1016/j.radonc.2009.12.006.

No association between SNPs regulating TGF- β 1 secretion and late radiotherapy toxicity to the breast: Results from the RAPPER study §

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Abstract

Background and purpose—Several small studies have reported associations between *TGFB1* single nucleotide polymorphisms (SNPs), considered to increase secretion of TGF- β 1, and greater than 3-fold increases in incidence of fibrosis – an indicator of late toxicity after radiotherapy in breast cancer patients.

Materials and methods—Two SNPs in *TGFB1*, C-509T (rs1800469) and L10P (rs1800470), were genotyped in 778 breast cancer patients who had received radiotherapy to the breast. Late radiotherapy toxicity was assessed two years after radiotherapy using a validated photographic technique, clinical assessment and patient questionnaires.

Results—On photographic assessment, 210 (27%) patients showed some degree of breast shrinkage, whilst 45 (6%) patients showed marked breast shrinkage. There was no significant association of genotype at either of the *TGFB1* SNPs with any measure of late radiation toxicity.

Conclusion—This adequately powered trial failed to confirm previously reported increases in fibrosis with *TGFB1* genotype – any increase greater than 1.36 can be excluded with 95% confidence. Similar frequent failures to replicate associations with candidate genes have been resolved using genome-wide association scans: this methodology detects common, low risk alleles but requires even larger patient numbers for adequate statistical power.

Keywords

Radiotherapy; Breast; Genetics; *TGFB1*; Breast cancer

RAPPER (Radiogenomics: Assessment of Polymorphisms for Predicting the Effects of Radiotherapy), a component project within GENEPI, is a large multi-centred UK collaboration studying the relationship between late radiotherapy morbidity and genetic

§On behalf of the Cambridge Breast IMRT Trial Investigators.

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variation measured by single nucleotide polymorphisms (SNPs) [1]. The Cambridge Breast Intensity Modulated Radiotherapy (IMRT) study has contributed over 50% of patient blood DNA samples to RAPPER [2].

The *TGFB1* gene encodes Transforming Growth Factor-beta1 (TGF- β 1), a pro-fibrotic cytokine, which stimulates differentiation of fibroblasts and production of extracellular matrix and inhibits epithelial repair [3]. Transforming Growth Factor exists in three different isoforms, of which TGF- β 1 is predominant in human plasma [4]. TGF- β 1 is synthesised as a large immature precursor molecule with a signal peptide, enabling extracellular secretion. A large circulating pool of latent TGF- β 1 is available for mobilisation after a triggering event [5], such as ionizing radiation [5-8].

The early phase of fibrogenesis after irradiation may be considered as a wound-healing response with up-regulation of pro-inflammatory cytokines [3], such as TGF- β 1. Endothelial cell killing leading to vascular damage and macrophage activation both contribute to tissue hypoxia, which in turn perpetuates fibrosis [9-11]. Normal epithelia may undergo epithelial to mesenchymal transition (EMT) in response to irradiation and TGF- β 1, which could also contribute to fibrosis [12]. The role of TGF- β 1 in the radiation response may be multi-factorial – related to development of fibrosis, extracellular signalling, induction of apoptosis and inhibition of proliferation in response to DNA damage [8,13].

TGF- β 1 has been implicated in the development of fibrosis in lung cancer patients [14-17]. Some studies have reported a correlation between elevated serum TGF- β 1 levels and increased fibrosis in breast cancer patients [18]. However, others have not found such associations [19]. Several small studies have reported a correlation between SNPs in the *TGFB1* gene and fibrosis, one form of late radiotherapy toxicity, in breast cancer patients [20-22]. In addition, a study of prostate cancer patients found significant associations between TGF genotype and certain toxicity end-points [23]. However, a recent study found no significant association between genotype and late clinical radiosensitivity in patients treated for gynaecological tumours [24]. A recent review of genetic variants and radiation toxicity included studies of the *TGFB1* gene and highlighted the methodological issues involved [25]. The SNPs that have been studied in the *TGFB1* gene include C-509T (rs1800469) and T+29C (rs1800470 encoding Leu10Pro; previously known as rs1982073), which are in strong linkage disequilibrium (LD) with each other such that the minor allele of C-509T (the T allele) and the minor C allele of L10P, encoding proline Pro10 tend to be inherited together.

We sought to confirm reports indicating that SNPs associated with this increased secretion of TGF- β 1 are also associated with increased radiation toxicity in a large number of patients who have been recruited to the RAPPER study. The RAPPER study is designed to look for an association between genetic variation and the development of radiation toxicity in several cancer types [26]. We assessed radiation toxicity using photographic assessment of breast shrinkage and clinical assessment of telangiectasia, induration of the breast, breast oedema and pigment change. Patient-reported pain and hyper-sensitivity of the treated breast were also studied.

Materials and methods

The patient characteristics and radiotherapy technique used in the Cambridge Breast IMRT trial have been previously described [2]. In this study 778 women, with operable unilateral histological-confirmed breast cancer (T1-3, N0-1, M0 at presentation) or ductal carcinoma in situ (DCIS) requiring radiotherapy after complete macroscopic excision of the tumour by breast conserving surgery (no implants), were treated either with forward-planned IMRT or

standard 2-field radiotherapy. A standard plan consisting of paired tangents was produced for all trial patients. Dose–volume histograms were then created for all patients for evaluation of dose homogeneity. Patients with significant dose inhomogeneities were randomised to either standard breast radiotherapy (285 patients in the control arm) or IMRT (286 in the interventional arm). Those patients with satisfactory dose homogeneity were not randomised, but treated with standard radiotherapy and followed up as per the randomised patients (207 patients).

The planning target volume (PTV) was the whole breast with a 1 cm margin. A dose of 40 gray (Gy) in 15 fractions, 5 days a week over 3 weeks was prescribed to the ICRU 50 reference point. An energy of 6 MV was used in most patients, but mixed energies of 6 and 15 MV photons were used when required in patients with larger separations. Nodal radiotherapy and a tumour bed boost were administered according to local protocols [2]. A boost was given to all patients except those deemed at low risk with all of the following: age >50 years, T1 stage, Grade 1 or 2, absence of lymph node metastases (including micrometastases), no lymphovascular space invasion, margins ≥ 5 mm (or 2 mm if anterior or deep margin and no more tissue to take). The tumour bed with a 2 cm radial margin was treated to a dose of 9 Gy in 3 fractions prescribed to the 100% isodose over 3 days by a direct electron field using 6–15 MeV electrons, depending on the skin–chest wall separation.

Data were collected on potentially confounding factors such as age at randomisation, smoking history, body mass index, use of tamoxifen and chemotherapy, breast boost, breast volume, ethnicity and co-morbidity such as diabetes mellitus, cardiovascular and peripheral vascular disease. Ethical approval was obtained from the Cambridge Research Ethics Committee. Written informed consent was obtained for all patients. All patients provided a blood sample.

Study end-points

The primary end-point of the Cambridge Breast IMRT trial was photographic assessment of late cosmetic effects. Frontal photographs of both breasts were taken after primary surgery and before the start of radiotherapy, and repeated at 2 years after treatment. Two photographs were taken, one with the hands resting on the hips, the other with the arms raised above the head. Follow-up photographs were terminated in the case of local tumour relapse, further breast surgery, declining health or patient refusal. Changes in breast shrinkage were scored on a 3-point scale (none/minimal = 1, mild = 2, marked = 3) by three observers comparing the photographs taken at 2 years with the baseline images reaching a consensus score. A multidisciplinary team of seven clinicians (four oncologists, a radiographer, a surgeon and a breast care nurse) were involved in the assessment of the clinical photographs, a panel of three being present at any one time. This method had been validated and shown to be as sensitive but quicker than using three independent scorers with re-scoring of discrepancies and final resolution through discussion [27–29]. In this study photographic assessment of breast shrinkage due to radiotherapy was used as the end-point to assess any relationship with genotype.

Clinical assessment was made by a trained specialist radiographer (JW) at a follow-up appointment at 2 years after completion of radiotherapy. The initial 70 patients were assessed by an oncologist (CC). A specialist radiographer JW was trained in clinical assessment by CC during this time period. She then assessed the remainder of the patients. The treated breast was examined for the presence of breast oedema, telangiectasia, change in pigmentation, and palpable induration of the breast. Induration of the breast was defined as hardening of the breast tissue and was the clinical end-point used to assess the pathological process of fibrosis. Each of the secondary clinical end-points was graded 0–3 (none, a little,

quite a bit, very much) on the scale used in the START trials [27,28]. Pigmentation change was scored from 0 to 2 according to the Late Effects of Normal Tissue-Subjective Objective Management Analytical (LENT-SOMA) score [30,31].

Patients completed validated quality of life questionnaires at baseline, 6 months and 2 years after the completion of radiotherapy. In this genotyping study, results from the EORTC BR23 questionnaire were used to assess pain and hyper-sensitivity in the treated breast at 2 years following radiation treatment [32]. Pain and oversensitivity experienced in the last week were scored on a scale from 1 to 4 (not at all, a little, quite a bit and very much). Patients recorded any medication that was prescribed for the management of breast pain.

Genotyping

DNA was extracted from the blood samples by Tepnel Life Sciences (www.tepnel.com). High-quality DNA was extracted from >99% of samples, with an average yield of 195 ± 67 μ g (range 22–494 μ g). All samples were genotyped using the Taqman 7900HT Sequence Detection System according to manufacturer's instructions. Each assay was carried out using 10 ng DNA in a 5 μ L reaction using Taqman Universal PCR Master Mix (Applied Biosystems, Warrington, United Kingdom), forward and reverse-primers and FAM- and VIC-labelled probes designed by Applied Biosystems (ABI Assay-by-Design). The sequences of the forward- and reverse-primers for L10P were AGGCGTCAGCACCAGTAG and GGAGAGCAATTCTTACAGGTGTCT, respectively. The sequence of the Vic-labelled probe was CAGCAGCGGCAGCA whilst that of the Fam-labelled probe was CAGCAGCAGCAGCA. For C-509T the sequences of the forward- and reverse-primers were CTGGGAAACAAGGTAGGAGAAGAG and GGAGAGCAATTCTTACAGGTGTCT. The sequence of the Vic-labelled probe was ACACCTGAAGGATGGA and that of the Fam-labelled probe was CACCTGAGGGATGGA. For both SNPs 40 cycles of PCR were carried out at 60 °C. All assays were carried out in 384-well arrays and each plate contained negative controls. In addition, 5% of all samples were duplicated and deviations of the genotype frequencies from those expected under Hardy–Weinberg equilibrium (HWE) were assessed by χ^2 tests, for quality control. Genotypes were determined using Allelic Discrimination Sequence Detection software (Applied Biosystems). DNA samples that did not give a clear genotype result at the first attempt were not repeated. The call rates were 96.1% and 99.6% for L10P and C-509T, respectively, concordance between duplicate samples was 100% and the genotype distributions did not differ from those expected under HWE.

Statistical analysis

Polychotomous logistic regression was used to relate genotype with photographic and clinical assessment scores. Polychotomous logistic regression, also known as ordinal response regression, was considered most appropriate for analysing the data as the dependent variables (toxicity end-points) take multiple ordered values. No numeric relationship is assumed between these grades; it is only assumed that lower grades correspond to milder reactions. Patients are assumed to have a decreasing risk of developing a specific grade of reaction when going from rare allele homozygous to heterozygous and to common allele homozygous. This form of regression effectively looks for a trend both down and across a table of toxicity score against genotype. In addition, the mean scores for each toxicity end-point according to genotype were calculated and the resulting *P* value for trend was derived using linear regression. The trend test assumes an increasing or decreasing risk of toxicity with increasing number of rare alleles. Whilst polychotomous logistic regression provides good power to detect association across a range of genetic models, the parameter estimates do not have an easily interpretable meaning. In order to aid their interpretation we

dichotomized the variables. End-points were dichotomized by combining scores of 0 and 1 as one group and 2 as the other group.

If the effect of genotype occurred above a dose-threshold, rather than with increasing dose, it could be postulated that the administration of a radiotherapy boost may be an interacting factor. If such a model was correct, a patient's genotype may not have an effect on the incidence of toxicity at lower doses. Genotype may only become important if they received a dose over a certain threshold. We therefore investigated possible interactions with radiotherapy boost using the log likelihood functions and calculating the change in model deviance when interaction terms were incorporated.

Results

Late clinical assessment data and genotype were available for 778 participants; photographic data were available for 703 study participants. Patient questionnaire data were available in 681 patients. Analysis was performed on all 778 patients; therefore some patients had missing values for some end-points. On photographic assessment, 210 patients (27%) showed some degree of breast shrinkage, whilst 45 patients (6%) showed marked breast shrinkage. The mean age of the patients was 59 years (range 26–83). Table 1 shows the results of clinical assessment of toxicity.

Table 2 shows the correlation between the different toxicity end-points used in the study. It can be seen that in many cases there is a significant correlation between two end-points, but the correlation coefficient is relatively low. It appears that patients who experience one toxicity end-point are more likely to experience other aspects of toxicity. Unsurprisingly there is a significant correlation between patient-reported breast pain and oversensitivity of the breast ($R = 0.53$; $P < 0.0001$).

As expected, breast volume was significantly correlated with the development of both acute and late effects. Patients with larger breast volumes were significantly more likely to experience breast shrinkage assessed by photographs, telangiectasia, breast oedema, pigmentation, breast pain and changes in skin sensation. Increased toxicity was seen in all end-points for patients who received a radiotherapy boost. There were statistically significant differences in photographic assessment of radiotherapy shrinkage ($P = 0.008$), clinical assessment of breast oedema ($P = 0.001$) and of breast induration ($P < 0.001$) and patient-reported breast pain ($P = 0.02$) between patients who did and did not receive a boost. The influence of both breast volume and boost on the development of late toxicity validates the sensitivity of the end-points used in this study.

Table 3 shows the results of polychotomous logistic regression for each of the late toxicity outcomes with genotype at the L10P and C-509T SNPs. There are no significant associations of any late outcome with genotype. Table 4 shows the mean scores for each toxicity end-point according to genotype and the resulting P value for trend. The trend test assumes an increasing or decreasing risk of toxicity with increasing number of rare alleles. The P values for trend are similar to those calculated using polychotomous logistic regression. Using this trend test, there were no significant associations between genotype at L10P or C-509T and any of the toxicity end-points. After dichotomizing the variables, there were also no significant associations between genotype and any of the toxicity end-points assessed.

Specifically, we saw no significant association of either SNP with mean induration score (a clinical measure of fibrosis) although the mean induration score of patients who were homozygotes for the minor Proline allele of L10P was higher (1.34) than the mean induration scores of those who carried the Leucine allele (1.28). After dichotomizing the

end-point induration, patients who were heterozygous for the rare allele variant of L10P had a 1.04-fold increased risk of fibrosis (95% CI 0.83–1.30). A similar pattern was observed with C-509T with an odds ratio of 1.08 per allele (95% CI 0.85–1.36). This is to be expected given the strong linkage disequilibrium between L10P and C-509T, which have a correlation coefficient (r^2) of 0.84 in these patients.

Potential confounding factors such as age at randomisation, smoking history, body mass index, use of tamoxifen and chemotherapy, breast boost, breast volume, ethnicity and co-morbidity such as diabetes mellitus, cardiovascular and peripheral vascular disease were not associated with TGF β 1 genotype. We found no evidence for an interaction of genotype with the administration of a radiotherapy boost.

Discussion

This large, purpose designed study has been unable to confirm recent reports of significant associations between the *TGFB1* (C-509T) SNP and increased risk of radiation fibrosis [20–22]. We did not find a significant association between either of the correlated *TGFB1* SNPs, L10P (rs1800470) or C-509T (rs1800469), with the development of induration, assessed by the clinician. Induration of the breast is the clinical end-point used to assess the pathological process of fibrosis. Furthermore, we found no significant association of these SNPs with photographic assessment of radiotherapy shrinkage, clinician's assessment of other late toxicity end-points or patient-reported symptoms.

C-509T is in the promoter region of *TGFB1* but is not within any putative promoter regulatory elements and Leu10Pro is in the signal peptide. The two SNPs have been reported to act by altering the rate of secretion of *TGFB1* and hence the circulating levels of mature protein [33–36]. However, not all studies have demonstrated such a relationship [37] – the presence, in the circulation, of both latent and activated forms may complicate the interpretation of studies on circulating levels.

Andreassen et al. found an increased risk of fibrosis in 41 patients who underwent post-mastectomy radiotherapy in patients with the Pro/Pro genotype in codon 10 and the T/T genotype in position –509 [21]. In addition, a similar SNP association with risk of subcutaneous fibrosis was found in 26 breast cancer patients and 26 matched controls [38]. However, these results were not replicated in a further study by the same group [39]. In a combined analysis of two published studies [20,22] of adjuvant radiotherapy to the breast (a total of 236 patients) it was reported that CT heterozygotes for C-509T had a 3-fold increased risk and TT homozygotes had a 15-fold increased risk of fibrosis following radiotherapy compared with CC homozygotes [22].

In our study 283 patients had induration of grade 2. The highly significant association of increased induration with breast boost shows that this is a sensitive end-point. We had 99% power to detect a 3-fold increase in incidence of fibrosis or induration for carriers of the T allele of the C-509T SNP, as reported in the combined analysis [22], with a type I error rate of 0.05. It remains possible that these SNPs do influence the development of late toxicity, but with much smaller effect sizes than reported in the previous small studies. This study enables the exclusion of a greater than 1.36-fold increased incidence of induration, associated with the T allele of C-509T, with >95% confidence. In order to have a 90% power to detect an effect size of 1.36 with a type I error rate of 0.05, assuming a minor allele frequency of 30% for the C-509T SNP and the same incidence of induration as in this study, a sample size of 1400 patients would be required.

Normal tissue reaction such as induration and telangiectasia develop gradually after a latency period [40–42]. However, there is evidence that late effects at 2 years are predictive

of those at 5 years [43]. The follow-up of this study is currently 2 years and therefore it is likely that not all patients will have expressed their final level of toxicity. This study is on-going and follow-up will continue on these patients, so re-analysis will be possible at 5 years.

Conclusion

Our finding emphasises the need for trials of sufficient size to have rigorous statistical power. It also illustrates a common problem, found with most other human genetic traits; that is, an iterative candidate gene approach is not an efficient methodology for discovering genetic determinants of variation. In traits where the common-variant-common-disease model holds, or at least contributes, well-designed genome-wide association scan (GWAS) methodology has proved more efficient and definitive at finding the common variants [26].

Acknowledgments

Dr. Gill Barnett is funded by a fellowship from Cancer Research UK and The Royal College of Radiologists. The Breast Cancer Campaign provides funding for the Cambridge Breast IMRT Trial radiographer, Jennifer Wilkinson and Cancer Research UK funds the RAPPER study. Alison Dunning is funded by CR-UK and Paul Pharoah is a Senior Clinical Research Fellow of CR-UK. Dr. Neil Burnet and Dr. Charlotte Coles are supported by the NIHR Cambridge Biomedical Research Centre. Professor Catharine West also has ECMC support for the work.

The Cambridge Breast IMRT Trial investigators include Dr. A.M. Moody, Dr. C.B. Wilson, Ms. C. McQuade, Professor G.C. Wishart, Mrs. N. Twyman and Dr. A.C.F. Hoole.

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Table 1

The frequency of adverse effects by photographic and clinical assessment.

Late toxicity end-point	Frequency of clinical score (%)					Total
	0	1	2	3	4	
Photographic assessment of breast shrinkage	N/A	448 (63.7)	210 (29.9)	45 (6.4)	N/A	703
Telangiectasia	657 (84.4)	63 (8.1)	42 (5.4)	16 (2.1)	N/A	778
Any induration	135 (17.4)	356 (45.8)	222 (28.5)	65 (8.4)	N/A	778
Breast oedema	444 (57.1)	205 (26.3)	100 (12.9)	29 (3.7)	N/A	778
Pigment change	549 (81.3)	62 (9.2)	64 (9.5)	N/A	N/A	675
Breast pain ^a	N/A	340 (50.1)	294 (43.4)	37 (5.5)	7 (1.0)	678
Oversensitivity of the breast	N/A	414 (60.8)	228 (33.5)	30 (4.4)	9 (1.3)	681

^a Only 20 patients took analgesia for breast pain, 1 took evening primrose oil, 4 took paracetamol, 4 took ibuprofen, 2 used combination of paracetamol and ibuprofen, 2 patients used medication for neuropathic pain and 7 patients required a weak opioid.

Table 2

The correlation between the different variables used in the study correlation coefficients are indicated for each pair of end-points with the corresponding *P* values in parenthesis.

	Photographic breast shrinkage	Telangiectasia	Breast induration	Breast oedema	Pigment change	Breast pain
Photographic breast shrinkage	1.00					
Telangiectasia	0.19 (<0.0001)	1.00				
Breast induration	0.18 (<0.0001)	0.05 (0.21)	1.00			
Breast oedema	0.28 (<0.0001)	0.22 (<0.0001)	0.13 (0.0004)	1.00		
Pigment change	0.11 (<0.0001)	0.13 (0.0005)	0.07 (0.07)	0.28 (<0.0001)	1.00	
Breast pain	0.14 (0.0005)	0.04 (0.25)	0.06 (0.14)	0.11 (0.006)	0.006 (0.89)	1.00
Oversensitivity of the breast	0.15 (0.0002)	0.085 (0.027)	0.077 (0.045)	0.16 (<0.0001)	0.037 (0.37)	0.53 (<0.0001)

Table 3
Association of toxicity end-points with genotype at L10P and C-509T using regression analysis.

Late toxicity end-point	L10P (rs1800470)		C-509T (rs1800469)	
	Regression coefficient ^a (95% CI)	P value	Regression coefficient ^a (95% CI)	P value
Photographic breast shrinkage	0.08 (−0.15, 0.31)	0.48	0.01 (−0.23, 0.25)	0.92
Telangiectasia	−0.05 (−0.35, 0.26)	0.77	−0.19 (−0.51, 0.13)	0.23
Breast induration	0.03 (−0.17, 0.22)	0.79	0.03 (−0.18, 0.24)	0.77
Breast oedema	−0.04 (−0.24, 0.17)	0.74	0.00 (−0.21, 0.22)	0.98
Pigment change	−0.03 (−0.32, 0.26)	0.85	−0.17 (−0.49, 0.14)	0.27
Breast pain	−0.18 (−0.42, 0.04)	0.11	−0.14 (−0.38, 0.10)	0.26
Oversensitivity of the breast	−0.16 (−0.40, 0.08)	0.18	−0.12 (−0.37, 0.13)	0.35

CI, confidence intervals.

^aRegression coefficients are from polychotomous logistic regression.

Table 4

Mean scores of toxicity end-points and genotype at L10P and C-509T.

Late toxicity end-point	L10P (rs1800470)				C-509T (rs1800469)			
	Mean scores (standard deviation) by genotype				Mean scores (standard deviation) by genotype			
	Common allele homozygote (TT)	Heterozygote (TC)	Rare allele homozygote (CC)	<i>P</i> trend ^a	Common allele homozygote (CC)	Heterozygote (CT)	Rare allele homozygote (TT)	<i>P</i> trend ^a
Photographic breast shrinkage	1.40 (0.60)	1.45 (0.62)	1.42 (0.56)	0.64	1.42 (0.62)	1.44 (0.63)	1.37 (0.49)	0.87
Telangiectasia	0.20 (0.57)	0.26 (0.64)	0.16 (0.58)	0.93	0.27 (0.67)	0.25 (0.64)	0.15 (0.55)	0.25
Breast induration	1.28 (0.80)	1.28 (0.87)	1.34 (0.91)	0.64	1.28 (0.82)	1.26 (0.86)	1.38 (0.94)	0.68
Breast oedema	0.68 (0.88)	0.59 (0.81)	0.69 (0.86)	0.64	0.65 (0.87)	0.61 (0.83)	0.68 (0.83)	0.86
Pigment change	0.29 (0.63)	0.27 (0.61)	0.3 (0.66)	0.99	0.32 (0.66)	0.24 (0.57)	0.31 (0.68)	0.29
Breast pain	1.62 (0.66)	1.55 (0.63)	1.49 (0.61)	0.08	1.61 (0.67)	1.55 (0.62)	1.52 (0.61)	0.15
Oversensitivity of the breast	1.53 (0.70)	1.39 (0.58)	1.45 (0.54)	0.06	1.53 (0.72)	1.39 (0.57)	1.5 (0.58)	0.09

^aThe mean scores for each toxicity end-point according to genotype were calculated and the resulting *P* value for trend was derived using linear regression.