Validation of tumor markers in central nervous system germ cell tumors by real-time reverse transcriptase polymerase chain reaction using formalin-fixed paraffin-embedded tissues

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Abstract. The therapeutic protocols for treatment of germinomas and non-germinomatous germ cell tumors (NGGCTs) are completely different, so it is important to distinguish pure germinomas from NGGCTs. As it can be difficult to diagnose by morphology alone, immunohistochemistry (IHC) has been widely used as an ancillary test to improve diagnostic accuracy. However, IHC has limitations due to the misinterpretation of results or the aberrant loss of immunoreactivity. However, real-time RT-PCR has certain advantages over IHC, including its quantitative nature. The aim of our study was to evaluate the usefulness of real-time RT-PCR on formalin-fixed paraffin-embedded (FFPE) tissue blocks for the diagnosis of germ cell tumors of the central nervous system. We selected eight markers of germ cell tumors using a literature search, and validated them using real-time RT-PCR. Among them, POU5F1, NANOG and TGFB2 were statistically significant (P=0.05) in multiple comparisons (MANOVA) of three groups (pure germinomas, mature teratomas and malignant germ cell tumors). Two-group (pure germinomas and NGGCTs) discriminant analysis achieved a 70.0% success rate in cross-validation. We concluded that real-time RT-PCR using FFPE tissue has adequate validating power comparable to IHC in the diagnosis of central nervous system germ cell tumors; therefore, when IHC is not available, not conclusive or not informative, RT-PCR is a potential alternative to a repeat biopsy.

Introduction

With the emergence of versatile imaging modalities, stereotactic or endoscopic biopsy has become the gold standard for confirming brain tumors (1). However, in small biopsies, the tissue obtained may not truly represent the whole lesion due to the small amount of tissue compared to the excisional specimen or may not be diagnostic due to artifacts such as electrocauterization or pinching (2,3). As the therapeutic protocols for treatment of germinomas and non-germinomatous germ cell tumors (NGGCTs) are completely different, it is important to distinguish pure germinomas from NGGCTs (4-7). Immunohistochemistry (IHC) is broadly used as an essential ancillary test in order to reach an exact diagnosis. Despite the importance of IHC, in everyday practice problems such as misinterpretation of results and aberrant loss of immunoreactivity are frequently encountered. Reverse transcriptase polymerase reaction (RT-PCR) emerged in the mid-1980s and became routine in most laboratories due to its reliable amplification of target DNA sequences with relatively small quantities of starting deoxyribonucleic acid (DNA) or ribonucleic acid (RNA) (8). In addition to dichotomous results from IHC, the recent introduction of more advanced real-time RT-PCR has enabled the quantitative analysis of levels of expression for target markers. Moreover, this quantitative analysis may reflect submorphologic alterations that were not observed in the usual hematoxylin and eosin (H&E) stained sections. To evaluate the possibilities of this new type of analysis as an alternative ancillary test for the diagnosis of brain germ cell tumors, we performed real-time RT-PCR on formalin-fixed paraffin-embedded (FFPE) tissues. After a meticulous literature search, we selected eight markers of germ cell tumors with relatively consistent expression levels for real-time RT-PCR. These markers were POU5F1, TGFB2, SLUG, NANOG, TWIST2 and cytokeratin 8, 18 and 19 (9-16). We performed this new PCR technique on the above molecular markers and statistically analyzed the results using SPSS.

Materials and methods

Patients and tissue samples. Samples of 30 pure germinomas and 30 NGGCTs from 60 patients (one case from each patient) treated between 1997 and 2010 were retrieved from the Pathology Department archive at Yonsei University Health System. Twenty-nine were biopsied tissue and 31 were removed tumor specimens <3 cm^3. Two neuropathologists independently reviewed the slides and a consensus diagnosis was reached. Among the 30 NGGCTs, 22 were teratomas

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Table I. Primers for RT-PCR.

Genes	Forward	Reverse
POU5F1	gaaggatgtggtccgagtgt	gcctcaaaatcctctcgttg
NANOG	aacaatcaggcctggaacag	gaatttggctggaactgcat
CK8	acatcgagatcgccacctac	tcatgttctgcatcccagac
CK18	gagtatgaggccctgctgaa	agtcctcgccatcttccag
CK19	cgatgtgcgagctgatagtg	gtaggtggcaatctcctgct
SLUG	gagcatttgcagacaggtca	ttggagcagtttttgcactg
TWIST2	agatccagacgctcaagctg	attgtccatctcgtcgctct
TGFB2	gtctcttgccggaatgtcag	ttctccacaaactcccttgg

(including 2 immature teratomas), 4 were mixed germ cell tumors, 3 were yolk sac tumors and 1 was a choriocarcinoma. This study was approved by the Institutional Review Board (IRB) of Medicine of Yonsei University Severance Hospital, Seoul, Korea (IRB no. 4-2010-0060). Written informed consent was obtained from the patient or their family.

Quantitative real-time polymerase chain reaction. It is well-known that mRNAs of POU5F1 (Oct4), NANOG, KLF4, podoplanin and CD133 are preferentially expressed in fresh germinoma cells. On the contrary, mRNAs of cytokeratin 8, 18 and 19, LOX, PDGFR-α, TGF-β2, TWIST2 and SLUG are preferentially expressed in fresh tissue of NGGCTs (9-16).

Total RNA was isolated from FFPE blocks using the RNeasy Mini kit (cat no. 74404; Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. The cDNA was synthesized from 1 μg total RNA using Maxime RT PreMix (cat no. 25082; Intron Biotechnology, Seongnam, Korea) and anchored oligo(dt)15-primers (Table I). Real-time PCR was performed with the 7300 Real-Time PCR System™ (Applied Biosystems, Foster City, CA, USA) using the SYBR-Green PCR Master mix™ (cat no. 4309155; Applied Biosystems). The relative amount of target mRNA was determined using an established comparative threshold (Ct) method which normalizes target mRNA Ct values (17,18).

Statistical analyses. Traditionally, central nervous system germ cell tumors are divided into two major categories: germinomas and NGGCTs. NGGCTs can be further classified into (mature) teratomas and malignant germ cell tumors (19,20). We decided to apply these clinically oriented classification schemes to our data. For the detailed analysis, the tumors were divided into three groups (germinoma, teratoma and malignant germ cell tumor) and multivariate analysis of variance (MANOVA) was performed, which mitigates type I errors by multiple executions of ANOVA. This was followed by Bonferroni's method, which is known to be the most conservative and rigorous post-hoc test (21). The second discriminant analysis, which

Table II. Real-time RT-PCR results with diagnoses.

Case no.	Diagnosis	-ΔCt							
		CK8	CK18	CK19	POU5F1	SLUG	TWIST2	NANOG	TGFB2
3	Germinoma	-1.00767	0.583733	3.1581	2.424867	2.928667	2.728567	0.160567	4.296267
4	Germinoma	-1.20563	0.062767	2.099033	2.1905	1.639533	2.247333	-0.47423	3.642767
5	Germinoma	-1.53637	0.4567	1.697667	1.770933	2.131733	0.959	-0.4425	2.548417
6	Germinoma	-1.26707	-0.3116	4.9747	3.157233	3.237883	1.983667	0.023467	2.055133
10	Germinoma	-0.61357	0.420467	3.748467	1.429633	2.4846	3.551067	-0.17223	4.019233
19	Germinoma	-1.80027	-0.0502	2.819367	3.1176	6.890433	2.367267	-0.1965	2.066167
25	Germinoma	-2.01793	0.1391	3.33635	2.813967	0.021	1.496	-0.15773	1.96435
30	Germinoma	-1.28947	0.222	3.647267	1.7797	2.9049	2.654633	-0.37153	2.929233
31	Germinoma	-0.91313	-0.1749	4.1586	1.8723	2.1411	4.339533	-0.41943	3.01015
32	Germinoma	-1.49033	-0.27653	2.265567	1.376767	2.294367	2.013667	-0.1783	3.4891
33	Germinoma	-0.71483	0.374533	3.924933	2.009667	2.552317	2.771633	0.1473	5.273133
34	Germinoma	-1.00013	0.088833	2.4746	1.2874	3.055033	2.977167	-0.2569	3.181633
36	Germinoma	-0.90683	0.248233	2.469467	0.774867	2.639667	2.3003	-0.58293	2.695167
37	Germinoma	-1.293	-0.0163	-1.46337	1.658967	2.091483	2.3596	-0.2831	2.763867
39	Germinoma	-0.96793	0.0619	2.906967	1.2681	3.35675	3.23295	-0.5723	4.198167
41	Germinoma	-1.27347	-0.0463	2.360067	1.5673	3.2893	2.154217	-0.26743	2.801267
42	Germinoma	-1.1849	-0.127	2.815167	1.555633	5.996267	1.946567	-0.1945	1.9058
43	Germinoma	-0.93637	-0.44463	2.503733	0.5257	2.811433	4.255733	-0.6514	2.715433
46	Germinoma	-0.94077	-0.08003	2.6776	1.671033	1.922033	3.029	-0.1714	2.993533
48	Germinoma	-0.6794	0.550333	2.4182	1.775867	2.7911	2.5145	-0.0121	3.2365
49	Germinoma	-3.75823	-0.13203	2.246167	1.576967	2.865433	2.229033	-0.6652	2.569933
51	Germinoma	-1.772	-0.6808	2.899133	1.651567	3.1057	0.1736	-0.73093	2.021867

Table II. Continued.

Case no.	Diagnosis	-ΔCt							
		CK8	CK18	CK19	POU5F1	SLUG	TWIST2	NANOG	TGFB2
53	Germinoma	-1.8097	3.1919	1.75775	4.6743	0.0057	-1.31955	-1.5189	-1.0174
54	Germinoma	-1.8803	-0.22143	4.283967	1.767267	3.798033	-0.60637	-0.2241	2.142567
55	Germinoma	-1.12427	-0.8882	2.013133	0.695333	2.231667	1.710467	-0.7146	1.5868
56	Germinoma	-0.98222	0.006817	1.682783	0.936517	3.266917	1.399283	-0.48538	2.39995
58	Germinoma	-1.81733	-0.29917	0.794033	2.607233	3.0084	-0.23167	0.021533	1.448
59	Germinoma	-1.5597	0.946667	2.217167	2.163467	2.356033	-0.3791	1.4012	2.582267
60	Germinoma	0.4351	1.689817	5.40495	1.764	4.1474	1.191567	0.717367	5.635633
61	Germinoma	1.5121	2.651867	3.720733	2.787567	3.843467	3.007367	0.347067	4.022367
1	Teratoma	-1.07187	-0.70867	3.0858	1.129633	2.354633	2.3666	-0.09417	2.830833
2	Teratoma	-1.6625	-0.86807	1.814267	0.590033	3.173367	1.6501	-0.72787	1.7907
7	Teratoma	-1.12157	0.432533	2.5073	0.925967	2.133133	3.5036	-0.07157	2.2191
8	Teratoma	-1.44467	0.222333	3.000033	1.5308	2.9343	3.301667	-0.33973	3.408767
9	Teratoma	-1.31247	-0.48737	2.254767	1.684533	3.200467	2.651533	-0.0423	3.598033
12	Teratoma	-1.87033	-0.69117	3.420467	0.957933	2.895583	2.862033	-0.6477	1.105167
13	Teratoma	-1.25463	-0.30903	1.9494	4.691867	2.084567	2.371967	0.1059	3.905633
14	Teratoma	-1.97357	-0.8182	2.065767	0.7972	2.469367	2.001533	-0.81503	2.009
15	Teratoma	-1.38267	-0.61597	1.654133	0.755233	1.703667	3.108133	-0.7875	2.261667
16	Teratoma	-1.4731	-0.7013	1.943367	1.276167	3.5551	2.948067	-0.31197	2.078833
20	Teratoma	-1.61087	-0.1822	2.167967	1.559167	2.1812	2.932933	-0.62773	2.031667
22	Teratoma	-1.3678	-0.13687	0.9671	2.1591	2.291433	1.764633	0.115333	2.19
23	Teratoma	-1.37627	-0.69413	1.152733	3.317	3.149033	2.660333	0.224967	3.3006
24	Teratoma	-1.41153	-0.45553	1.588433	2.0532	1.410533	2.252383	-0.60863	3.128183
29	Teratoma	1.282933	-0.1224	2.2679	1.433067	2.6873	3.822	-0.11127	3.803967
40	Teratoma	-1.1878	-0.38887	1.2398	0.7109	2.194233	2.3635	-0.91057	2.754533
44	Teratoma	-1.08037	-0.06357	2.776667	2.188433	3.361033	3.089467	0.006633	3.884633
45	Teratoma	0.130333	0.920367	3.375967	2.8968	5.285783	6.428283	1.3523	4.379433
47	Teratoma	-1.0455	-0.07473	2.403467	2.575933	1.921033	4.4185	0.2723	4.507967
57	Teratoma	-3.137	1.4696	-1.36997	0.387	0.0166	-3.07047	-0.1507	-0.88195
38	Yolk sac tumor	-1.09567	-0.49573	1.4576	1.361367	2.6421	3.2136	-0.3545	2.309
64	Yolk sac tumor	2.416467	1.855333	1.884967	5.106767	6.263133	5.5104	4.040667	7.086167
66	Yolk sac tumor	-0.09807	0.286367	2.2285	2.852	3.970367	3.043033	1.0156	3.815367
52	Immature teratoma	-1.1578	0.2049	2.841367	1.405367	2.389333	1.3699	-0.01897	3.195667
62	Immature teratoma	-0.75343	0.008867	2.5339	1.036367	4.4318	0.947167	-0.05707	2.646733
50	Choriocarcinoma	-2.2934	0.7866	5.193633	3.832033	3.904167	4.649167	1.286167	9.081467
18	Mixed germ cell tumor	-1.21753	-0.75473	2.5435	1.165033	1.8842	2.1788	-0.48403	2.282267
21	Mixed germ cell tumor	-0.5728	0.787433	3.155833	1.9952	3.1615	2.639433	-0.25033	3.699633
28	Mixed germ cell tumor	-0.89637	0.7643	3.350433	3.8734	3.6043	3.508167	0.945867	2.677467
35	Mixed germ cell tumor	-0.95173	0.184167	3.3223	1.782833	2.628617	4.334433	-0.46857	3.272267

Table III. Multivariate analysis of variance (MANOVA).

Variables	Sum of square	Degree of freedom	Square	F-value	P-value
POU5F1	7.220	2	3.610	3.619	0.033
TWIST2	18.172	2	9.086	4.216	0.020
NANOG	6.030	2	3.015	5.913	0.005
TGFB2	15.393	2	7.697	3.713	0.030

Table IV. Multivariate analysis of variance (MANOVA) with Bonferroni's post-hoc test.

Variables	Diagnosis A[a]	Diagnosis B[a]	Remainder (A-B)	SE[b]	P-value	95% CI Upper	95% CI Lower
POU5F1	2	3	-1.106911	0.4123491	0.028	-2.124047	-0.089775
TWIST2	1	3	-1.666061	0.5841194	0.018	-3.106901	-0.225222
NANOG	1	3	-0.947197	0.2841285	0.005	-1.648053	-0.246340
	2	3	-0.909328	0.2948037	0.009	-1.636517	-0.182140
TGFB2	1	3	-1.438711	0.5728709	0.045	-2.851804	-0.025618
	2	3	-1.543901	0.5943946	0.036	-3.010087	-0.077716

[a]1, pure germinoma; 2, mature teratoma; 3, malignant germ cell tumor. [b]SE, standard error.

Table V. Discriminant analysis - predicted classification by resultant discriminant function.

	Diagnosis[a]	Predicted group 1	Predicted group 2	Sum
Original				
Frequency	1	22.0	8.0	30
	2	6.0	24.0	30
Percentage	1	73.3	26.7	100
	2	20.0	80.0	100
Cross-validation				
Frequency	1	20.0	10.0	30
	2	8.0	22.0	30
Percentage	1	66.7	33.3	100
	2	26.7	73.3	100

[a]1, germinoma; 2, non-germinomatous germ cell tumor.

enables two-group discrimination, was applied to the above two major categories. Statistical analysis was performed with SPSS version 12.0 software (SPSS, Chicago, IL, USA). P-values of <0.05 were considered to indicate a statistically significant difference.

Results

Real-time RT-PCR. All samples were successfully analyzed by real-time RT-PCR (Table II). The majority of RNA samples were in good condition. They had optical density ratios 260/280 between 1.8 and 2.0 (22).

Multiple comparisons. Retrieved cases were assigned to three groups as follows: pure germinoma, mature teratoma and malignant germ cell tumor (Table II). MANOVA was performed and Bonferroni's method was used as a post-hoc correction. The results are shown in Tables III and IV, respectively. Among the selected genes, POU5F1, TWIST2, NANOG and TGFB2 were statistically significant, and these results were consistent with the previous literature, which used conventional immunohistochemistry or other ancillary tests on germ cell tumors (9-16).

Two-group discriminant analysis. Retrieved cases were randomly assigned to two groups as follows: pure germinoma (group 1) and non-germinomatous germ cell tumor (group 2). Genes that were statistically significant in the previous MANOVA (POU5F1, TWIST2, NANOG and TGFB2) correctly discriminate 76.7% of original groups and 70% of cross-validation groups (Table V). In general, when discriminant functions classify >70% of cases correctly (hit ratio), it is considered to demonstrate good discriminating power (23).

Discussion

Real-time RT-PCR was introduced in the mid-1990s as a quantitative test for mRNA. Conventional IHC can be partly utilized as a semi-quantitative test for proteins, but there was no test for mRNA until this method emerged. Our primary aim was to introduce this quantitative test to FFPE-based pathology practice, particularly for the diagnosis of CNS germ cell tumors.

Data summarized in Table IV show markers that are useful to separate various germ cell tumors. POU5F1 is useful to discriminate mature teratomas from malignant germ cell tumors. TWIST2 is useful to discriminate pure germinomas from malignant germ cell tumors. NANOG and TGFB2 are useful to discriminate germ cell tumors with a benign clinical course from malignant germ cell tumors. Therefore, real-time RT-PCR may be applied more specifically to these particular situations. For example, in differentiating pure germinoma and malignant germ cell tumors, high -ΔCt of POU5F1 may be an indication of pure germinoma. For mature teratomas and malignant germ cell tumors, high -ΔCt of TWIST2 implies a malignant germ cell tumor. If one focuses on the benign vs. malignant problem, high -ΔCt of NANOG and TGFB2 are suggestive of malignancy. Although it is beyond the scope of this paper to determine the critical cut-off ΔCt value of the marker genes mentioned above, in difficult situations, the correct diagnosis may be made using a combination of the

selected genes. Discriminant analysis also correctly sorts 70% of pure germinomas from other tumor types (Table V). All of our results suggest that real-time RT-PCR of FFPE tissues is a useful method for quantitative analysis in the diagnosis of CNS germ cell tumors.

In our opinion, although real-time RT-PCR cannot completely replace conventional immunohistochemistry in small biopsy specimens due to its labor-intensiveness, real-time RT-PCR is a second-line adjunctive test when IHC results are not informative.

There are several papers comparing the results of real-time RT-PCR from fresh tissue and from FFPE tissue such as lymph node, breast and lung cancer tissues (24-26). Although this type of validation is more direct and confirmative, it is not easy to obtain fresh brain tissue without hindrance to the correct diagnosis, so we decided to indirectly compare data from RT-PCR to data from the published literature, and have concluded that our results are consistent with previous results.

In conclusion, our experiment statistically validates real-time RT-PCR in FFPE-based pathology practice by using multivariate methods in neuropathology, which is in agreement with other papers that utilized RT-PCR on other types of pathology (27-31). Additionally, we showed that mRNAs of germ cell tumors were robust in FFPE tissue for a period of approximately 10 years. Real-time RT-PCR may be an alternative ancillary test, particularly when conventional immunohistochemistry is not available or not sufficient to make a correct diagnosis. With further study, it could be used as a primary diagnostic ancillary test in the future, similar to IHC.

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